DESCRIPTION

ANTI-MPL ANTIBODY

Technical Field

5   The present invention relates to anti-Mpl antibodies.

Background Art

Thrombopoietin (TPO) is a factor that enhances the differentiation and maturation of megakaryocytes (platelet precursor
10 cells) from hemopoietic stem cells into platelets. TPO also functions as a cytokine with an important role in the regulation of platelet number. TPO is converted into its active form through the cleavage of a TPO precursor comprising 353 amino acids.

Mpl is a TPO receptor, and human Mpl molecules are known to exist
15 in two forms comprising 572 and 635 amino acids. The human Mpl gene sequence has already been analyzed (see Non-Patent Document 1 and GenBank accession No. NM_005373).

Most cytokine receptors dimerize upon ligand binding, and transduce signals into cells. It has been reported that TPO similarly
20 binds to its own specific receptor MPL, which leads to dimerization of the receptor, thereby transducing signals into cells and exerting physiological effects (see Non-Patent Document 2).

Antibodies exhibiting agonistic activity have been reported among those antibodies that bind to receptors having the above
25 features.

For example, an antibody against the erythropoietin (EPO) receptor has been reported to substitute for erythropoietin function. The monovalent form (Fab) of the antibody is capable of binding to the EPO receptor but is unable to transduce signals. Thus,
30 dimerization of the erythropoietin receptor via bivalent binding is assumed to be essential for signal transduction (see Non-Patent Document 3).

Antibodies that bind to Mpl and exhibit TPO agonistic activity have also been reported (see Non-Patent Documents 4 and 5). This
35 suggests that receptor dimerization is induced upon binding of a bivalent antibody with regards to MPL as well.

Meanwhile, a single-chain antibody (scFv) has been reported to exhibit TPO agonistic activity (see Patent Document 1). However, it has been revealed that, the underlying mechanism of scFv exhibiting TPO agonistic activity is that a part of scFv dimerizes (diabody) and this diabody becomes the actual active unit (see Patent Documents 2 to 4).

[Patent Document 1] US Patent No. 6342220

[Patent Document 2] WO 01/79494

[Patent Document 3] WO 02/33072

[Patent Document 4] WO 02/33073

[Non-Patent Document 1] Palacios et al., Cell, 1985, 41, 727-734

[Non-Patent Document 2] Souyri et al., Cell, 1990, 63, 1137-1147

[Non-Patent Document 3] Elliott, S. et al., J. Biol. Chem., 1996, 271(40), 24691-24697

[Non-Patent Document 4] Abe et al., Immunol. Lett., 1998, 61, 73-78

[Non-Patent Document 5] Bijia Deng et al., Blood, 1998, 92, 1981-1988

Disclosure of the Invention

Problems to Be Solved by the Invention

The present invention was achieved in view of the above circumstances. An objective of the present invention is to provide novel anti-Mpl antibodies having TPO agonistic activity.

Means to Solve the Problems

The present inventors performed exhaustive research to solve the above objective. The present inventors prepared and purified anti-human Mpl antibody VB22B, and established a single-chain antibody expression system using genetic engineering techniques. Specifically, the variable region of anti-human Mpl antibody was first cloned, and a diabody expression vector pCXND3-VB22B db for the anti-human Mpl antibody was prepared. This pCXND3-VB22B db vector was then used to generate an expression vector pCXND3-VB22B sc(Fv)2 for anti-human Mpl antibody sc(Fv)2. Anti-human Mpl sc(Fv)2 was expressed in CHO-DG44 cells using the expression vector pCXND3-VB22B sc(Fv)2, and then purified from the culture supernatant. In control experiments, VB22B diabody was transiently expressed in COS7 cells

using the above pCXND3-VB22B db vector, and then purified from the culture supernatant.

In addition, VB22B diabody and VB22B sc(Fv)2 were evaluated for their TPO-like agonistic activities. The results showed that VB22B diabody and VB22B sc(Fv)2 exhibit higher agonistic activities compared to VB22B IgG, and thus activities equivalent to or higher than that of the natural ligand, human TPO.

Furthermore, the present inventors succeeded in preparing five types of humanized VB22B sc(Fv)2. The TPO-like agonistic activity was also proven to be unaltered by humanization.

More specifically, the present invention provides the following (1) to (38):

(1) an antibody comprising a single-chain polypeptide having binding activity against TPO receptor (Mpl), wherein said antibody comprises two heavy chain variable regions and two light chain variable regions;

(2) the antibody of (1), wherein the two heavy chain variable regions and the two light chain variable regions are arranged in the order of heavy chain variable region, light chain variable region, heavy chain variable region, and light chain variable region from the N terminus of the single-chain polypeptide;

(3) the antibody of (1) or (2), wherein the two heavy chain variable regions and the two light chain variable regions are linked by linkers;

(4) the antibody of (3), wherein the linkers comprise 15 amino acids;

(5) a chimeric antibody that binds to Mpl;

(6) the antibody of (5), which is a humanized antibody;

(7) the antibody of (5) or (6), which is a minibody;

(8) an antibody that binds to soluble Mpl;

(9) an antibody that binds to human Mpl and monkey Mpl;

(10) an antibody having agonistic activity against human Mpl and monkey Mpl;

(11) an antibody whose binding activity to soluble Mpl is KD = 10^{-6} M or lower;

(12) an antibody whose binding activity to soluble Mpl is KD

$= 10^{-7}$ M or lower;

(13) an antibody whose TPO agonistic activity is EC50 = 100 nM or lower;

(14) an antibody whose TPO agonistic activity is EC50 = 30 nM or lower;

(15) an antibody whose TPO agonistic activity is EC50 = 10 nM or lower;

(16) an antibody which comprises a heavy chain variable region, wherein said heavy chain variable regions comprises CDR1, CDR2 and CDR3 consisting of an amino acid sequence of any one of:

[1] SEQ ID NOs: 3, 4, and 5

[2] SEQ ID NOs: 6, 7, and 8

[3] SEQ ID NOs: 9, 10, and 11

[4] SEQ ID NOs: 15, 16, and 17

[5] SEQ ID NOs: 18, 19, and 20

[6] SEQ ID NOs: 21, 22, and 23

[7] SEQ ID NOs: 24, 25, and 26

[8] SEQ ID NOs: 27, 28, and 29

[9] SEQ ID NOs: 30, 31, and 32

[10] SEQ ID NOs: 33, 34, and 35

[11] SEQ ID NOs: 36, 37, and 38

[12] SEQ ID NOs: 39, 40, and 41

[13] SEQ ID NOs: 42, 43, and 44

[14] SEQ ID NOs: 48, 49, and 50

[15] SEQ ID NOs: 51, 52, and 53

[16] SEQ ID NOs: 54, 55, and 56

[17] SEQ ID NOs: 57, 58, and 59;

(17) an antibody which comprises a light chain variable region, wherein said light chain variable region comprises CDR1, CDR2 and CDR3 consisting of an amino acid sequence of any one of:

[1] SEQ ID NOs: 60, 61, and 62

[2] SEQ ID NOs: 63, 64, and 65

[3] SEQ ID NOs: 78, 79, and 80

[4] SEQ ID NOs: 84, 85, and 86

[5] SEQ ID NOs: 93, 94, and 95

[6] SEQ ID NOs: 96, 97, and 98

[7] SEQ ID NOs: 102, 103, and 104

[8] SEQ ID NOs: 108, 109, and 110

[9] SEQ ID NOs: 111, 112, and 113

[10] SEQ ID NOs: 114, 115, and 116;

(18) an antibody that comprises a heavy chain variable region and a light chain variable region of any one of:

[1] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 3, 4, and 5, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 60, 61, and 62;

[2] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 6, 7, and 8, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 63, 64, and 65;

[3] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 9, 10, and 11, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 63, 64, and 65;

[4] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 15, 16, and 17, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 63, 64, and 65;

[5] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 18, 19, and 20, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 63, 64, and 65;

[6] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 21, 22, and 23, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 78, 79, and 80;

[7] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 24, 25, and 26, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 63, 64, and 65;

[8] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 27, 28, and 29, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 84, 85, and 86;

[9] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 30, 31, and 32, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequence consisting of SEQ ID NOs: 63, 64, and 65;

[10] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 33, 34, and 35, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 63, 64, and 65;

[11] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 36, 37, and 38, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 93, 94, and 95;

[12] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 39, 40, and 41, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 96, 97, and 98;

[13] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 42, 43, and 44, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 78, 79, and 80;

[14] a heavy chain variable region that comprises CDR1, CDR2,

and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 45, 46, and 47, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 102, 103, and 104;

5   [15] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 48, 49, and 50, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 63, 64, and 65;

10   [16] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 51, 52, and 53, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 108, 109, and 110,

15   [17] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 54, 55, and 56, and a light chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 111, 112, and 113;

20   [18] a heavy chain variable region that comprises CDR1, CDR2, and CDR3 comprising the amino acid sequences consisting of SEQ ID NOs: 57, 58, and 59, and a light chain variable region that comprises CDR1, CDR2, and CDR3 each comprising the amino acid sequences consisting of SEQ ID NOs: 114, 115, and 116;

25   (19) an antibody that comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 118;

(20) an antibody that comprises a light chain variable region comprising the amino acid sequence of SEQ ID NO: 120;

(21) an antibody that comprises a heavy chain variable region
30 comprising the amino acid sequence of SEQ ID NO: 118 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 120;

(22) an antibody comprising the amino acid sequence of SEQ ID NO: 122 or 264;

(23) an antibody that comprises a heavy chain variable region,
35 wherein said heavy chain variable region comprises FR1, FR2, FR3, and FR4 consisting of amino acid sequences of any one of:

[1] SEQ ID NOs: 230, 232, 234, and 236

[2] SEQ ID NOs: 265, 267, 269, and 271

[3] SEQ ID NOs: 279, 281, 283, and 285

[4] SEQ ID NOs: 298, 299, 300, and 301

[5] SEQ ID NOs: 298, 299, 306, and 301.

(24) an antibody comprising a light chain variable region, wherein said light chain variable region comprises FR1, FR2, FR3, and FR4 consisting of amino acid sequences of any one of:

[1] SEQ ID NOs: 239, 241, 243, and 245

[2] SEQ ID NOs: 272, 274, 276, and 278

[3] SEQ ID NOs: 302, 303, 304, and 305

[4] SEQ ID NOs: 302, 307, 308, and 305;

(25) an antibody that comprises a heavy chain variable region and a light chain variable region of any one of:

[1] a heavy chain variable region which comprises FR1, FR2, FR3, and FR4 having the amino acid sequences consisting of SEQ ID NOs: 230, 232, 234, and 236, and a light chain variable region which comprises FR1, FR2, FR3, and FR4 having the amino acid sequences consisting of SEQ ID NOs: 239, 241, 243, and 245;

[2] a heavy chain variable region which comprises FR1, FR2, FR3, and FR4 having the amino acid sequences consisting of SEQ ID NOs: 265, 267, 269, and 271, and a light chain variable region which comprises FR1, FR2, FR3, and FR4 having the amino acid sequences consisting of SEQ ID NOs: 272, 274, 276, and 278;

[3] a heavy chain variable region which comprises FR1, FR2, FR3, and FR4 having the amino acid sequences consisting of SEQ ID NOs: 279, 281, 283, and 285, and a light chain variable region which comprises FR1, FR2, FR3, and FR4 having the amino acid sequences consisting of SEQ ID NOs: 272, 274, 276, and 278;

[4] a heavy chain variable region which comprises FR1, FR2, FR3, and FR4 having the amino acid sequences consisting of SEQ ID NOs: 298, 299, 300, and 301, and a light chain variable region which comprises FR1, FR2, FR3, and FR4 having the amino acid sequences consisting of SEQ ID NOs: 302, 303, 304, and 305;

[5] a heavy chain variable region which comprises FR1, FR2, FR3, and FR4 having the amino acid sequences consisting of SEQ ID NOs:

298, 299, 306, and 301, and a light chain variable region which comprises FR1, FR2, FR3, and FR4 having the amino acid sequences consisting of SEQ ID NOs: 302, 307, 308, and 305;

(26) an antibody that comprises a heavy chain variable region, wherein said heavy chain variable region comprises the amino acid sequence of SEQ ID NO: 229, 256, 262, 289, or 295;

(27) an antibody that comprises a light chain variable region, wherein said light chain variable region comprises the amino acid sequence of SEQ ID NO: 238, 258, 291, or 297;

(28) an antibody that comprises a heavy chain variable region and a light chain variable region of any one of:

[1] a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 229, and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 238;

[2] a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 256, and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 258;

[3] a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 262, and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 258;

[4] a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 289, and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 291;

[5] a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 295, and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 297;

(29) an antibody that comprises the amino acid sequence of SEQ ID NO: 2, 254, 260, 287, or 293;

(30) an antibody having an activity equivalent to that of an antibody of any one of (16) to (29), wherein said antibody comprises the amino acid sequence set forth in any one of (16) to (29), in which one or more amino acids have been substituted, deleted, added and/or inserted;

(31) an antibody that recognizes an epitope recognized by an antibody of any one of (16) to (30);

(32) an antibody that recognizes the region of amino acids 26

to 274 of human Mpl;

(33) an antibody of any one of (1) to (32), which has TPO agonistic activity;

(34) a polynucleotide encoding an antibody of any one of (1) to (33);

(35) a polynucleotide hybridizing to the polynucleotide of (34) under stringent conditions, wherein said polynucleotide encodes an antibody having activity equivalent to that of an antibody of any one of (1) to (33);

(36) a vector comprising the polynucleotide of (34) or (35);

(37) a host cell that carries the polynucleotide of (34) or (35), or the vector of (36); and

(38) a pharmaceutical composition comprising an antibody of any one of (1) to (33).

Brief Description of the Drawings

Fig. 1 demonstrates the strategy for preparing single-chain antibody sc(Fv)2.

Fig. 2 illustrates the assessment of VB22B sc(Fv)2 binding activity using an Mpl-expressing CHO cell line. Purified VB22B sc(Fv)2 was used.

Fig. 3 illustrates the assessment of VB22B antibody agonistic activity using BaF3-human Mpl.

Fig. 4 illustrates the assessment of VB22B antibody agonistic activity using BaF3-monkey Mpl.

Fig. 5 illustrates the assessment of VB22B antibody agonistic activity using M-07e.

Fig. 6 shows the amino acid sequences of anti-human Mpl antibodies (H chains) that exhibit higher agonistic activities when converted into minibodies.

Fig. 7 shows the amino acid sequences of anti-human Mpl antibodies (L chains) which exhibit higher agonistic activities when converted into minibodies.

Fig. 8 illustrates the binding activity assessment of AB317 diabody using Mpl-expressing CHO cells. Both VB22B diabody (solid line) and AB317 diabody (broken line) were obtained from COS7 culture

supernatants.

Fig. 9 illustrates the agnostic activity assessment of AB324 and AB317 diabodies using BaF3-human Mpl.

Fig. 10 illustrates the agnostic activity assessment of AB324 and AB317 diabodies using BaF3-monkey Mpl.

Fig. 11 illustrates the agnostic activity assessment of AB324 and AB317 diabodies using BaF3-mouse Mpl.

Fig. 12 shows the agonistic activities of diabodies in BaF3-human Mpl cells. The X-axis shows OD at 450/655 nm, and the Y-axis represents concentration.

Fig. 13 shows the agonistic activities of diabodies in BaF3-human Mpl (G305C) cells. The X-axis shows OD at 450/655 nm, and the Y-axis represents concentration.

Fig. 14 shows the agonistic activities of TA136 db and TA136 sc(Fv)2 in BaF3-human Mpl cells. The X-axis shows OD at 450/655 nm and the Y-axis represents concentration.

Fig. 15 shows the agonistic activities of TA136 db and TA136 sc(Fv)2 in BaF3-human Mpl (G305C) cells. The X-axis shows OD at 450/655 nm, and the Y-axis represents concentration.

Fig. 16 shows the agonistic activities of TA136 db and TA136 sc(Fv)2 in BaF3-human Mpl (C769T) cells. The X-axis shows OD at 450/655 nm, and the Y-axis represents concentration.

Fig. 17 shows the agonistic activities of TA136 db and TA136 sc(Fv)2 in BaF3-human Mpl (C823A) cells. The X-axis shows OD at 450/655 nm, and the Y-axis represents concentration.

Fig. 18 shows the positions of FRs and CDRs in humanized heavy chain sequences (hVB22B p-z, hVB22B g-e, hVB22B e, hVB22B u2-wz4, and hVB22B q-wz5:VH), and humanized light chain sequences (hVB22B p-z, hVB22B g-e, hVB22B e, hVB22B u2-wz4, and hVB22B q-wz5:VL).

Fig. 19 shows the TPO-like agonistic activities of murine VB22B sc(Fv)2, hVB22B e sc(Fv)2, and hVB22B g-e sc(Fv)2 in BaF3-human Mpl. The X-axis shows absorbance ratio (450 nm/655 nm), and the Y-axis represents concentration.

Fig. 20 shows the TPO-like agonistic activities of murine VB22B sc(Fv)2, hVB22B p-z sc(Fv)2, and hVB22B u2-wz4 sc(Fv)2 in BaF3-human Mpl. The X-axis shows absorbance ratio (450 nm/655 nm), and the Y-axis

represents concentration.

Fig. 21 shows the TPO-like agonistic activities of murine VB22B sc(Fv)2 and hVB22B q-wz5 sc(Fv)2 in BaF3-human Mpl. The X-axis shows absorbance ratio (450 nm/655 nm), and the Y-axis represents concentration.

Best Mode for Carrying Out the Invention

The present invention provides antibodies that bind to the TPO receptor (Mpl).

The antibodies of the present invention comprise all types of antibodies, including antibodies with modified amino acid sequences, such as minibodies, humanized antibodies, and chimeric antibodies; antibodies that have been modified by binding with other molecules (for example, polymers such as polyethylene glycol); and antibodies whose sugar chains have been modified.

Mpl of the present invention may be a mutant receptor. A mutant receptor of the present invention is usually a receptor that exists at a frequency lower than 50%, preferably lower than 20%, more preferably lower than 10%, and even more preferably lower than 1%. The frequency is generally calculated using randomly selected subjects. However, the frequency may vary depending on the country, area, sex, and such. Therefore, the frequency may also be calculated, for example, within a defined country or area, such as Japan, the United States, and Europe, or calculated for one sex. When there are two or more mutations in a receptor, the frequency may be calculated for multiple mutation sites or for any one of the mutation sites. Mutant receptors are preferably evaluated by a frequency as described above. However, mutant receptors can also be evaluated, for example, by their signal transducing ability and such. Specifically, for example, when two different receptors are present, the one with stronger transducing signals upon natural ligand-binding maybe be used as a non-mutant type receptor, and the one with weaker transducing signals as a mutant receptor.

In one embodiment, the mutant receptors of the present invention comprise receptors that are associated with disease onset. The phrase "mutant receptors associated with disease onset" means that

the loss of reactivity to a natural ligand becomes part of the reason that triggers disease onset. In the present invention, the mutant receptor may be a contributing factor, but not necessarily the sole factor triggering disease onset. Many reports have been previously published that describe the association of mutant receptors with disease onset. In addition to those that have been reported, associations of mutant receptors and disease onset can also be identified by statistical analysis methods (for example, correlation analyses). Correlation analyses, also called "case control studies", are well known to those skilled in the art (for example, Nishimura, Y., 1991, "Statistical analysis of polymorphisms", Saishin Igaku, 46:909-923; Oka, A. *et al.*, 1990, Hum. Mol. Genetics 8, 2165-2170; Ota, M. *et al.*, 1999, Am. J. Hum. Genet. 64, 1406-1410; Ozawa, A. *et al.*, 1999, Tissue Antigens 53, 263-268). For example, the correlation between a mutant receptor and a disease can be studied by computing the frequency of the mutant receptor in patients and healthy subjects, and testing whether the patient population has a higher mutant receptor frequency. Typically, differences in frequency are evaluated using the χ-test. χ is obtained by the equation $\chi^2 = \Sigma$(observed value – expected value)^2/expected value. A p value is obtained from the χ^2 value determined. Based on this p value, it can be determined whether there is a correlation between the mutant receptor and the disease. For example, when $p < 0.05$, the mutant receptor is considered to correlate with the disease. Mutant thrombopoietin (TPO) receptors have already been reported (Matthias Ballmaier *et al.*, 2001, BLOOD, 97 (1), 139; and others).

It is preferable that the antibodies of the present invention have agonistic activity against Mpl.

In a preferred embodiment, the antibodies of the present invention comprise, for example, minibodies.

The minibodies comprise antibody fragments lacking portions of the whole antibody (for example, whole IgG). The minibodies are not particularly limited as long as they have binding activity to their antigens. The minibodies of the present invention have higher activities compared to their corresponding whole antibodies. There are no particular limitations on the antibody fragments of the present

invention as long as they are portions of the whole antibody, and preferably contain heavy chain variable regions (VH) and/or light chain variable regions (VL). The amino acid sequences of VH or VL may contain substitutions, deletions, additions and/or insertions.

5   Furthermore, the antibody fragment may also lack portions of VH or/and VL, as long as it has binding ability to its antigen. In addition, the variable regions may be chimerized or humanized. Such antibody fragments include, for example, Fab, Fab', F(ab')$_2$, and Fv. An example of a minibody includes Fab, Fab', F(ab')$_2$, Fv, scFv

10 (single-chain Fv), diabody, and sc(Fv)2 (single-chain (Fv)2).

Herein, an "Fv" fragment is the smallest antibody fragment and contains a complete antigen recognition site and a binding site. The "Fv" fragment is a dimer (VH-VL dimer) in which a single VH and a single VL are strongly linked by a non-covalent bond. The three

15 complementarity-determining regions (CDRs) of each of the variable regions interact with each other to form an antigen-binding site on the surface of the VH-VL dimer. Six CDRs confer the antigen-binding site of an antibody. However, a single variable region (or a half of Fv containing only three CDRs specific to an antigen) alone is

20 also capable of recognizing and binding an antigen although its affinity is lower than the affinity of the entire binding site.

scFv contains the VH and VL regions of an antibody, and these regions exist on a single polypeptide chain. Generally, an Fv polypeptide further contains a polypeptide linker between VH and VL,

25 and therefore an scFv can form a structure required for antigen binding. See, Pluckthun "The Pharmacology of Monoclonal Antibodies" Vol. 113 (Rosenburg and Moore eds. (Springer Verlag, New York, pp. 269-315, 1994) for the review of scFv. In the present invention, linkers are not especially limited as long as they do not inhibit expression of

30 antibody variable regions linked at both ends of the linkers.

The term "diabody" refers to a bivalent antibody fragment constructed by gene fusion (Holliger P et al., 1993, Proc. Natl. Acad. Sci. USA 90: 6444-6448; EP 404,097; WO 93/11161 and others). Diabodies are dimers comprising two polypeptide chains, where each

35 polypeptide chain comprises a VL and a VH connected with a linker short enough to prevent interaction of these two domains, for example,

a linker of about five residues. The VL and VH encoded on the same polypeptide chain will form a dimer because the linker between them is too short to form a single-chain variable region fragment. As a result, the polypeptide chains form a dimer, and thus the diabody has two antigen binding sites.

sc(Fv)2 is a single-chain minibody produced by linking two units of VH and two units of VL with linkers and such (Hudson et al., 1999, J Immunol. Methods 231:177-189). sc(Fv)2 exhibits a particularly high agonistic activity compared to the whole antibody and other minibodies. sc(Fv)2 can be produced, for example, by linking two scFv molecules.

In a preferable antibody, the two VH units and two VL units are arranged in the order of VH, VL, VH, and VL ([VH]-linker-[VL]-linker-[VH]-linker-[VL]) beginning from the N terminus of a single-chain polypeptide.

The order of the two VH units and two VL units is not limited to the above arrangement, and they may be arranged in any order. Examples of the arrangements are listed below.

[VL]-linker-[VH]-linker-[VH]-linker-[VL]

[VH]-linker-[VL]-linker-[VL]-linker-[VH]

[VH]-linker-[VH]-linker-[VL]-linker-[VL]

[VL]-linker-[VL]-linker-[VH]-linker-[VH]

[VL]-linker-[VH]-linker-[VL]-linker-[VH]

The linkers to be used for linking the variable regions of an antibody comprise arbitrary peptide linkers that can be introduced by genetic engineering, synthetic linkers, and linkers disclosed in, for example, Holliger, P. et al., Protein Engineering, 9(3), 299-305, 1996. Peptide linkers are preferred in the present invention. There are no limitations as to the length of the peptide linkers. The length can be selected accordingly by those skilled in the art depending on the purpose, and is typically 1-100 amino acids, preferably 3-50 amino acids, more preferably 5-30 amino acids, and even more preferably 12-18 amino acids (for example, 15 amino acids).

For example, such peptide linkers include:

Ser

Gly·Ser

Gly·Gly·Ser

Ser·Gly·Gly

Gly·Gly·Gly·Ser

Ser·Gly·Gly·Gly

5   Gly·Gly·Gly·Gly·Ser

Ser·Gly·Gly·Gly·Gly

Gly·Gly·Gly·Gly·Gly·Ser

Ser·Gly·Gly·Gly·Gly·Gly

Gly·Gly·Gly·Gly·Gly·Gly·Ser

10   Ser·Gly·Gly·Gly·Gly·Gly·Gly

(Gly·Gly·Gly·Gly·Ser)$_n$

(Ser·Gly·Gly·Gly·Gly)$_n$

where n is an integer of 1 or larger. The lengths and sequences of peptide linkers can be selected accordingly by those skilled in the

15   art depending on the purpose.

In an embodiment of the present invention, a particularly preferable sc(Fv)2 includes, for example, the sc(Fv)2 below.
[VH]-peptide linker (15 amino acids)-[VL]-peptide linker (15 amino acids)-[VH]-peptide linker (15 amino acids)-[VL]

20   Synthetic linkers (chemical crosslinking agents) include crosslinking agents routinely used to crosslink peptides, for example, N-hydroxy succinimide (NHS), disuccinimidyl suberate (DSS), bis(succinimidyl) suberate (BS^3), dithiobis(succinimidyl propionate) (DSP), dithiobis(succinimidyl propionate) (DTSSP),

25   ethylene glycol bis(succinimidyl succinate) (EGS), ethylene glycol bis(sulfosuccinimidyl succinate) (sulfo-EGS), disuccinimidyl tartrate (DST), disulfosuccinimidyl tartrate (sulfo-DST), bis[2-(succinimidoxycarbonyloxy)ethyl] sulfone (BSOCOES), and bis[2-(succinimidoxycarbonyloxy)ethyl] sulfone (sulfo-BSOCOES).

30   These crosslinking agents are commercially available.

In general, three linkers are required to link four antibody variable regions together. The linkers to be used may be of the same type or different types. In the present invention, a preferable minibody is a diabody, even more preferably, an sc(Fv)2. Such a

35   minibody can be prepared by treating an antibody with an enzyme, for example, papain or pepsin, to generate antibody fragments, or by

constructing DNAs encoding those antibody fragments and introducing them into expression vectors, followed by expression in an appropriate host cell (see, for example, Co, M. S. *et al.*, 1994, J. Immunol. 152, 2968-2976; Better, M. and Horwitz, A. H., 1989, Methods Enzymol. 178, 476-496; Pluckthun, A. and Skerra, A., 1989, Methods Enzymol. 178, 497-515; Lamoyi, E., 1986, Methods Enzymol. 121, 652-663; Rousseaux, J. *et al.*, 1986, Methods Enzymol. 121, 663-669; Bird, R. E. and Walker, B. W., 1991, Trends Biotechnol. 9, 132-137).

An antibody having exceedingly high agonistic activity can be prepared by reducing the molecular weight of a full-length antibody, particularly by converting it into an sc(Fv)2.

In a preferred embodiment, the antibodies of the present invention comprise modified antibodies, such as chimeric antibodies and humanized antibodies that bind to Mpl. These modified antibodies can be produced by known methods.

Chimeric antibodies are antibodies prepared by combining sequences derived from different animal species, and include for example, antibodies comprising the heavy chain and light chain variable regions of a murine antibody, and the heavy chain and light chain constant regions of a human antibody. Chimeric antibodies can be prepared by known methods. For example, a DNA encoding the V region of an antibody is linked to a DNA encoding the C region of a human antibody, and the construct is inserted into an expression vector and introduced into a host to produce chimeric antibodies.

Humanized antibodies are also referred to as "reshaped human antibodies". Such a humanized antibody is obtained by transferring the complementarity-determining region (CDR) of an antibody derived from a non-human mammal, for example mouse, to the complementarity-determining region of a human antibody, and the general gene recombination procedure for this is also known (see European Patent Application No. 125023 and WO 96/02576).

Specifically, a DNA sequence designed to link a murine antibody CDR to the framework region (FR) of a human antibody can be synthesized by PCR, using primers prepared from several oligonucleotides containing overlapping portions of both CDR and FR terminal regions (see methods described in WO 98/13388).

The human antibody framework region to be linked by CDR is selected in order to form a favorable antigen-binding site in the complementarity-determining region. Amino acids of the framework region in the antibody variable region may be substituted, as necessary, for the complementarity-determining region of the reshaped human antibody to form a suitable antigen-binding site (Sato, K. *et al.*, 1993, Cancer Res. 53, 851-856).

The constant region of a human antibody is used as the constant region of a chimeric antibody or humanized antibody. For example, Cγ1, Cγ2, Cγ3, and Cγ4 can be used as the H chain, and Cκ and Cλ can be used as the L chain. The human antibody constant region may be modified to improve the antibody or the stability of the antibody production.

Generally, chimeric antibodies comprise the variable region of an antibody from a non-human mammal and the constant region derived from a human antibody. On the other hand, humanized antibodies comprise the complementarity-determining region of an antibody from a non-human mammal, and the framework region and constant region derived from a human antibody.

In addition, after a chimeric antibody or a humanized antibody is prepared, amino acids in the variable region (for example, FR) and the constant region may be replaced with other amino acids, and such.

The origin of the variable regions in chimeric antibodies or that of the CDRs in humanized antibodies is not particularly limited, and may be derived from any type of animal. For example, sequences of murine antibodies, rat antibodies, rabbit antibodies, camel antibodies may be used.

In general, it is difficult to chimerize or humanize an antibody without losing the agonistic activity of the original antibody. Nevertheless, the present invention succeeded in preparing humanized antibodies having agonistic activity equivalent to that of the original murine antibody.

A preferred humanized antibody of the present invention is an antibody comprising a heavy chain variable region that comprises the amino acid sequence of SEQ ID NO: 229 (humanized heavy chain sequence:

hVB22B p-z VH), SEQ ID NO: 256 (humanized heavy chain sequence: hVB22B g-e VH), SEQ ID NO: 262 (humanized heavy chain sequence: hVB22B e VH), SEQ ID NO: 289 (humanized heavy chain sequence: hVB22B u2-wz4 VH), or SEQ ID NO: 295 (humanized heavy chain sequence: hVB22B q-wz5 VH); or an antibody comprising a light chain variable region that comprises the amino acid sequence of SEQ ID NO: 238 (humanized light chain hVB22B p-z VL), SEQ ID NO: 258 (humanized light chain hVB22B g-e VL or hVB22B e VL), SEQ ID NO: 291 (humanized light chain hVB22B u2-wz4 VL), or SEQ ID NO: 297 (humanized light chain hVB22B q-wz5 VL). In particular, a preferred antibody is an antibody comprising a heavy chain variable region and a light chain variable region of any one of (1) to (5) indicated below:

(1) a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 229, and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 238;

(2) a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 256, and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 258;

(3) a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 262, and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 258;

(4) a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 289, and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 291; and

(5) a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 295, and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 297.

Such antibodies include, for example, antibodies comprising the amino acid sequence of SEQ ID NO: 2, 254, 260, 287, or 293 (humanized sc(Fv)2 sequence (hVB22B p-z sc(Fv)2, hVB22B g-e sc(Fv)2, hVB22B e sc(Fv)2, hVB22B u2-wz4, or hVB22B q-wz5).

The nucleotide sequence of hVB22B p-z VH is shown in SEQ ID NO: 228; the nucleotide sequence of hVB22B g-e VH is shown in SEQ ID NO: 255; the nucleotide sequence of hVB22B e VH is shown in SEQ ID NO: 261; the nucleotide sequence of hVB22B u2-wz4 VH is shown in SEQ ID NO: 288; the nucleotide sequence of hVB22B q-wz5 VH is shown in SEQ

ID NO: 294; the nucleotide sequence of hVB22B p-z VL is shown in SEQ ID NO: 237; the nucleotide sequences of hVB22B g-e VL and hVB22B e VL are shown in SEQ ID NO: 257; the nucleotide sequence of hVB22B u2-wz4 VL is shown in SEQ ID NO: 290; and the nucleotide sequence of hVB22B q-wz5 VL is shown in SEQ ID NO: 296.

In the amino acid sequence of SEQ ID NO: 229 (humanized heavy chain sequence: hVB22B p-z VH), SEQ ID NO: 256 (humanized heavy chain sequence: hVB22B g-e VH), SEQ ID NO: 262 (humanized heavy chain sequence: hVB22B e VH), SEQ ID NO: 289 (humanized heavy chain sequence: hVB22B u2-wz4 VH), or SEQ ID NO: 295 (humanized heavy chain sequence: hVB22B q-wz5 VH),

amino acids 31-35 correspond to CDR1;

amino acids 50-66 correspond to CDR2;

amino acids 99-107 correspond to CDR3;

amino acids 1-30 correspond to FR1;

amino acids 36-49 correspond to FR2;

amino acids 67-98 correspond to FR3; and

amino acids 108-118 correspond to FR4.

In the amino acid sequence of SEQ ID NO: 238 (humanized light chain sequence: hVB22B p-z VL), SEQ ID NO: 258 (humanized light chain sequence: hVB22B g-e VL or hVB22B e VL), SEQ ID NO: 291 (humanized light chain sequence: hVB22B u2-wz4 VL), or SEQ ID NO: 297 (humanized light chain sequence: hVB22B q-wz5 VL),

amino acids 24-39 correspond to CDR1;

amino acids 55-61 correspond to CDR2;

amino acids 94-102 correspond to CDR3;

amino acids 1-23 correspond to FR1;

amino acids 40-54 correspond to FR2;

amino acids 62-93 correspond to FR3; and

amino acids 103-112 correspond to FR4.

In the present invention, SEQ ID NOs of the CDRs and FRs in the hVB22B p-z VH sequence are shown below:

hVB22B p-z VH: FR1/SEQ ID NO: 230

hVB22B p-z VH: CDR1/SEQ ID NO: 36

hVB22B p-z VH: FR2/SEQ ID NO: 232

hVB22B p-z VH: CDR2/SEQ ID NO: 37

hVB22B p-z VH: FR3/SEQ ID NO: 234

hVB22B p-z VH: CDR3/SEQ ID NO: 38

hVB22B p-z VH: FR4/SEQ ID NO: 236.

In the present invention, SEQ ID NOs of the CDRs and FRs in the

5 hVB22B p-z VL sequence are shown below:

hVB22B p-z VL: FR1/SEQ ID NO: 239

hVB22B p-z VL: CDR1/SEQ ID NO: 93

hVB22B p-z VL: FR2/SEQ ID NO: 241

hVB22B p-z VL: CDR2/SEQ ID NO: 94

10 hVB22B p-z VL: FR3/SEQ ID NO: 243

hVB22B p-z VL: CDR3/SEQ ID NO: 95

hVB22B p-z VL: FR4/SEQ ID NO: 245.

In the present invention, SEQ ID NOs of the CDRs and FRs in the

hVB22B g-e VH sequence are shown below:

15 hVB22B g-e VH: FR1/SEQ ID NO: 265

hVB22B g-e VH: CDR1/SEQ ID NO: 36

hVB22B g-e VH: FR2/SEQ ID NO: 267

hVB22B g-e VH: CDR2/SEQ ID NO: 37

hVB22B g-e VH: FR3/SEQ ID NO: 269

20 hVB22B g-e VH: CDR3/SEQ ID NO: 38

hVB22B g-e VH: FR4/SEQ ID NO: 271.

In the present invention, SEQ ID NOs of the CDRs and FRs in the

hVB22B g-e VL sequence are shown below:

hVB22B g-e VL: FR1/SEQ ID NO: 272

25 hVB22B g-e VL: CDR1/SEQ ID NO: 93

hVB22B g-e VL: FR2/SEQ ID NO: 274

hVB22B g-e VL: CDR2/SEQ ID NO: 94

hVB22B g-e VL: FR3/SEQ ID NO: 276

hVB22B g-e VL: CDR3/SEQ ID NO: 95

30 hVB22B g-e VL: FR4/SEQ ID NO: 278.

In the present invention, SEQ ID NOs of the CDRs and FRs in the

hVB22B e VH sequence are shown below:

hVB22B e VH: FR1/SEQ ID NO: 279

hVB22B e VH: CDR1/SEQ ID NO: 36

35 hVB22B e VH: FR2/SEQ ID NO: 281

hVB22B e VH: CDR2/SEQ ID NO: 37

hVB22B e VH: FR3/SEQ ID NO: 283

hVB22B e VH: CDR3/SEQ ID NO: 38

hVB22B e VH: FR4/SEQ ID NO: 285.

In the present invention, SEQ ID NOs of the CDRs and FRs in the

5   hVB22B e VL sequence are shown below:

hVB22B e VL: FR1/SEQ ID NO: 272

hVB22B e VL: CDR1/SEQ ID NO: 93

hVB22B e VL: FR2/SEQ ID NO: 274

hVB22B e VL: CDR2/SEQ ID NO: 94

10   hVB22B e VL: FR3/SEQ ID NO: 276

hVB22B e VL: CDR3/SEQ ID NO: 95

hVB22B e VL: FR4/SEQ ID NO: 278.

In the present invention, SEQ ID NOs of the CDRs and FRs in the

hVB22B u2-wz4 VH sequence are shown below:

15   hVB22B u2-wz4 VH: FR1/SEQ ID NO: 298

hVB22B u2-wz4 VH: CDR1/SEQ ID NO: 36

hVB22B u2-wz4 VH: FR2/SEQ ID NO: 299

hVB22B u2-wz4 VH: CDR2/SEQ ID NO: 37

hVB22B u2-wz4 VH: FR3/SEQ ID NO: 300

20   hVB22B u2-wz4 VH: CDR3/SEQ ID NO: 38

hVB22B u2-wz4 VH: FR4/SEQ ID NO: 301.

In the present invention, SEQ ID NOs of the CDRs and FRs in the

hVB22B u2-wz4 VL sequence are shown below:

hVB22B u2-wz4 VL: FR1/SEQ ID NO: 302

25   hVB22B u2-wz4 VL: CDR1/SEQ ID NO: 93

hVB22B u2-wz4 VL: FR2/SEQ ID NO: 303

hVB22B u2-wz4 VL: CDR2/SEQ ID NO: 94

hVB22B u2-wz4 VL: FR3/SEQ ID NO: 304

hVB22B u2-wz4 VL: CDR3/SEQ ID NO: 95

30   hVB22B u2-wz4 VL: FR4/SEQ ID NO: 305.

In the present invention, SEQ ID NOs of the CDRs and FRs in the

hVB22B q-wz5 VH sequence are shown below:

hVB22B q-wz5 VH: FR1/SEQ ID NO: 298

hVB22B q-wz5 VH: CDR1/SEQ ID NO: 36

35   hVB22B q-wz5 VH: FR2/SEQ ID NO: 299

hVB22B q-wz5 VH: CDR2/SEQ ID NO: 37

hVB22B q-wz5 VH: FR3/SEQ ID NO: 306

hVB22B q-wz5 VH: CDR3/SEQ ID NO: 38

hVB22B q-wz5 VH: FR4/SEQ ID NO: 301.

In the present invention, SEQ ID NOs of the CDRs and FRs in the hVB22B q-wz5 VL sequence are shown below:

hVB22B q-wz5 VL: FR1/SEQ ID NO: 302

hVB22B q-wz5 VL: CDR1/SEQ ID NO: 93

hVB22B q-wz5 VL: FR2/SEQ ID NO: 307

hVB22B q-wz5 VL: CDR2/SEQ ID NO: 94

hVB22B q-wz5 VL: FR3/SEQ ID NO: 308

hVB22B q-wz5 VL: CDR3/SEQ ID NO: 95

hVB22B q-wz5 VL: FR4/SEQ ID NO: 305.

The CDRs and FRs in the hVB22B p-z sequence, hVB22B g-e sequence, hVB22B e sequence, hVB22B u2-wz4 sequence, and hVB22B q-wz5 sequence are shown in Fig. 18.

In other embodiments, preferred humanized antibodies of the present invention include:

humanized antibodies comprising a heavy chain variable region which has FR1, 2, 3, and 4 comprising amino acid sequences of any one of (1) to (5) indicated below:

(1) SEQ ID NOs: 230, 232, 234, and 236 (hVB22B p-z: H chain FR1, 2, 3, and 4),

(2) SEQ ID NOs: 265, 267, 269, and 271 (hVB22B g-e: H chain FR1, 2, 3, and 4),

(3) SEQ ID NOs: 279, 281, 283, and 285 (hVB22B e: H chain FR1, 2, 3, and 4),

(4) SEQ ID NOs: 298, 299, 300, and 301 (hVB22B u2-wz4: H chain FR1, 2, 3, and 4), and

(5) SEQ ID NOs: 298, 299, 306, and 301 (hVB22B q-wz5: H chain FR1, 2, 3, and 4);

humanized antibodies comprising a light chain variable region which has FR1, 2, 3, and 4 comprising amino acid sequences of any one of (1) to (4) listed below:

(1) SEQ ID NOs: 239, 241, 243, and 245 (hVB22B p-z: L chain FR1, 2, 3, and 4),

(2) SEQ ID NOs: 272, 274, 276, and 278 (hVB22B g-e or hVB22B e: L

chain FR1, 2, 3, and 4),

(3) SEQ ID NOs: 302, 303, 304, and 305 (hVB22B u2-wz4: L chain FR1, 2, 3, and 4), and

(4) SEQ ID NOs: 302, 307, 308, and 305 (hVB22B q-wz5: L chain FR1,

5   2, 3, and 4);

humanized antibodies comprising a heavy chain variable region which has CDR1, 2 and 3 comprising amino acid sequences according to the SEQ ID NOs listed below:

SEQ ID NOs: 36, 37, and 38 (hVB22B p-z, hVB22B g-e, hVB22B e, hVB22B

10   u2-wz4, or hVB22B q-wz5: H chain CDR1, 2, and 3); and

humanized antibodies comprising a light chain variable region which has CDR1, 2 and 3 comprising amino acid sequences according to the SEQ ID NOs listed below:

SEQ ID NOs: 93, 94, and 95 (hVB22B p-z hVB22B g-e, hVB22B e, hVB22B

15   u2-wz4, or hVB22B q-wz5: L chain CDR1, 2, and 3).

In yet another preferred embodiment, preferred humanized antibodies of the present invention include:

humanized antibodies comprising heavy chain and light chain variable regions of any one of (1) to (5) indicated below.

20   (1) a heavy chain variable region which comprises FR1, 2, 3, and 4 comprising the amino acid sequences of SEQ ID NOs: 230, 232, 234, and 236, respectively, and a light chain variable region which comprises FR1, 2, 3, and 4 comprising the amino acid sequences of SEQ ID NOs: 239, 241, 243, and 245, respectively;

25   (2) a heavy chain variable region which comprises FR1, 2, 3, and 4 comprising the amino acid sequences of SEQ ID NOs: 265, 267, 269, and 271, respectively, and a light chain variable region which comprises FR1, 2, 3, and 4 comprising the amino acid sequences of SEQ ID NOs: 272, 274, 276, and 278, respectively;

30   (3) a heavy chain variable region which comprises FR1, 2, 3 and 4 comprising the amino acid sequences of SEQ ID NOs: 279, 281, 283, and 285, respectively, and a light chain variable region which comprises FR1, 2, 3, and 4 comprising the amino acid sequences of SEQ ID NOs: 272, 274, 276, and 278, respectively;

35   (4) a heavy chain variable region which comprises FR1, 2, 3, and 4 comprising the amino acid sequences of SEQ ID NOs: 298, 299, 300,

and 301, and a light chain variable region which comprises FR1, 2, 3, and 4 comprising the amino acid sequences of SEQ ID NOs: 302, 303, 304, and 305, respectively;

(5) a heavy chain variable region which comprises FR1, 2, 3, and 4 comprising the amino acid sequences of SEQ ID NOs: 298, 299, 306, and 301, respectively, and a light chain variable region which comprises FR1, 2, 3, and 4 comprising the amino acid sequences of SEQ ID NOs: 302, 307, 308, and 305, respectively; and

humanized antibodies comprising heavy chain and light chain variable regions described below:

a heavy chain variable region which comprises CDR1, 2, and 3 comprising the amino acid sequences of SEQ ID NOs: 36, 37, and 38, respectively, and a light chain variable region which comprises CDR1, 2, and 3 comprising the amino acid sequences of SEQ ID NOs: 93, 94, and 95, respectively.

Chimeric antibodies and humanized antibodies exhibit lower antigenicity in the human body, and thus are expected to be useful when administered to humans for therapeutic purposes.

In one embodiment, the preferred antibodies of the present invention include antibodies that bind to soluble Mpl. The term "soluble Mpl" herein refers to Mpl molecules excluding those expressed on the cell membrane. A specific example of a soluble Mpl is an Mpl lacking the entire or a portion of the transmembrane domain. The transmembrane domain of human Mpl corresponds to amino acids 492-513 in SEQ ID NO: 123.

An antibody that binds to soluble recombinant Mpl can be used in detailed epitope analysis and kinetic analysis of receptor-ligand binding, as well as for assessing the blood concentration and dynamic behavior of the antibody in *in vivo* tests.

In one embodiment, the preferred antibodies of the present invention include antibodies having binding activity against both human and monkey Mpl. The present invention also provides antibodies having agonistic activity to human Mpl and monkey Mpl. Antibodies having agonistic activity to both human and monkey Mpl are expected to be highly useful since the dynamic behavior and *in vivo* effects of the antibody, which are generally difficult to determine in human

body, can be examined with monkeys.

Such antibodies may also have binding activity or agonistic activity against Mpl from animals other than humans and monkeys (for example, mice).

In addition, the antibodies of the present invention include antibodies with TPO agonistic activity (agonistic activity against Mpl) of EC50 = 100 nM or lower, preferably EC50 = 30 nM or lower, more preferably EC50 = 10 nM or lower.

The agonistic activity can be determined by methods known to those skilled in the art, for example, by the method described below. The sequences for human Mpl (Palacios et al., Cell 41:727-734, (1985); GenBank Accession NO. NM_005373), cynomolgus monkey Mpl (the nucleotide sequence and amino acid sequence are shown in SEQ ID NO: 164 and SEQ ID NO: 165, respectively), and mouse Mpl (GenBank Accession NO. NM_010823) are already known.

In addition, the present invention includes antibodies whose binding activities to soluble Mpl are KD = 10^{-6} M or lower, preferably KD = 10^{-7} M or lower.

In the present invention, whether the binding activity of an antibody to soluble recombinant Mpl is KD = 10^{-6} M or lower can be determined by methods known to those skilled in the art. For example, the activity can be determined using surface plasmon resonance with Biacore. Specifically, soluble MPL-Fc protein, soluble MPL protein, or epitope peptides recognized by antibodies are immobilized onto sensor chips. Reaction rate constant can be determined by assessing the interaction between the antibody and the soluble Mpl-Fc protein, the soluble Mpl protein, or the epitope peptide recognized by the antibody. The proteins to be immobilized on chips are not limited in particular, and include, for example, MG10 (from Gln213 to Ala231)-GST fusion protein and Mpl-IgG Fc fusion protein described in the Examples. Since the antibodies are divalent and have two antigen-binding sites, the binding activities of these antibodies may be determined as those for the monovalent or divalent antibodies, or for mixtures of both. Any of these can be used in the present invention.

The binding activity can be evaluated by ELISA (enzyme-linked

immunosorbent assays), EIA (enzyme immunoassays), RIA (radio immunoassays), or fluorescent antibody techniques. For example, in enzyme immunoassays, a sample containing a test antibody, such as purified antibody or culture supernatant of cells producing the test

5 antibody, is added to a plate coated with an antigen to which the test antibody can bind. After incubating the plate with a secondary antibody labeled with an enzyme such as alkaline phosphatase, the plate is washed and an enzyme substrate such as p-nitrophenyl phosphate is added. The antigen-binding activity can then be

10 evaluated by determining the absorbance.

There is no specific limitation as to the upper limit of the binding activity; for example, the upper limit may be set within a technically feasible range by those skilled in the art. However, the technically feasible range may expand with the advancement of

15 technology.

In an embodiment, the preferred antibodies of the present invention include antibodies recognizing epitopes that are recognized by any one of the antibodies indicated in (I) to (XII) below. The antibody of any one of (I) to (XII) is preferably a minibody.

20

(I) Antibody comprising a VH that has CDR1, 2, and 3 comprising the amino acid sequences according to SEQ ID NOs in any one of (1) to (17) indicated below (name of each antibody and the H chain CDR contained in the antibody are indicated inside the parentheses):

25 (1) SEQ ID NOs: 3, 4, and 5 (VA7: H chain CDR1, 2, and 3),

(2) SEQ ID NOs: 6, 7, and 8 (VA130 or VB17B: H chain CDR1, 2, and 3),

(3) SEQ ID NOs: 9, 10, and 11 (VA259: H chain CDR1, 2, and 3),

(4) SEQ ID NOs: 15, 16, and 17 (VB12B: H chain CDR1, 2, and 3),

30 (5) SEQ ID NOs: 18, 19, and 20 (VB140: H chain CDR1, 2, and 3),

(6) SEQ ID NOs: 21, 22, and 23 (VB33: H chain CDR1, 2, and 3),

(7) SEQ ID NOs: 24, 25, and 26 (VB45B: H chain CDR1, 2, and 3),

(8) SEQ ID NOs: 27, 28, and 29 (VB8B: H chain CDR1, 2, and 3),

(9) SEQ ID NOs: 30, 31, and 32 (VB115: H chain CDR1, 2, and 3),

35 (10) SEQ ID NOs: 33, 34, and 35 (VB14B: H chain CDR1, 2, and 3),

(11) SEQ ID NOs: 36, 37, and 38 (VB22B, VB4B, hVB22B p-z, hVB22B g-e,

hVB22B e, hVB22B u2-wz4 or hVB22B q-wz5: H chain CDR1, 2, and 3),

(12) SEQ ID NOs: 39, 40, and 41 (VB16: H chain CDR1, 2, and 3),

(13) SEQ ID NOs: 42, 43, and 44 (VB157: H chain CDR1, 2, and 3),

(14) SEQ ID NOs: 48, 49, and 50 (VB51: H chain CDR1, 2, and 3),

5 (15) SEQ ID NOs: 51, 52, and 53 (AB317: H chain CDR1, 2, and 3),

(16) SEQ ID NOs: 54, 55, and 56 (AB324: H chain CDR1, 2, and 3),

(17) SEQ ID NOs: 57, 58, and 59 (TA136: H chain CDR1, 2, and 3).

(II) Antibody comprising a VL which has CDR1, 2, and 3 comprising

10 the amino acid sequences according to SEQ ID NOs in any one of (1)

to (10) indicated below (name of each antibody and the L chain CDR

in the antibody are indicated inside the parentheses):

(1) SEQ ID NOs: 60, 61, and 62 (VA7: L chain CDR1, 2, and 3),

(2) SEQ ID NOs: 63, 64, and 65 (VA130, VA259, VB17B, VB12B, VB140,

15 VB45B, VB115, VB14B or VB51: L chain CDR1, 2, and 3),

(3) SEQ ID NOs: 78, 79, and 80 (VB33 or VB157: L chain CDR1, 2, and

3),

(4) SEQ ID NOs: 84, 85, and 86 (VB8B: L chain CDR1, 2, and 3),

(5) SEQ ID NOs: 93, 94, and 95 (VB22B, hVB22B p-z, hVB22B g-e, hVB22B

20 e, hVB22B u2-wz4 or hVB22B q-wz5: L chain CDR1, 2, and 3),

(6) SEQ ID NOs: 96, 97, and 98 (VB16: L chain CDR1, 2, and 3),

(7) SEQ ID NOs: 102, 103, and 104 (VB4B: L chain CDR1, 2, and 3),

(8) SEQ ID NOs: 108, 109, and 110 (AB317: L chain CDR1, 2, and 3),

(9) SEQ ID NOs: 111, 112, and 113 (AB324: L chain CDR1, 2, and 3),

25 (10) SEQ ID NOs: 114, 115, and 116 (TA136: L chain CDR1, 2, and 3).

(III) Antibody comprising a VH that comprises an amino acid sequence

of the SEQ ID NO in any one of (1) to (24):

(1) SEQ ID NO: 124 (VA7: VH),

30 (2) SEQ ID NO: 126 (VA130: VH),

(3) SEQ ID NO: 128 (VA259: VH),

(4) SEQ ID NO: 130 (VB17B: VH),

(5) SEQ ID NO: 132 (VB12B: VH),

(6) SEQ ID NO: 134 (VB140: VH),

35 (7) SEQ ID NO: 136 (VB33: VH),

(8) SEQ ID NO: 138 (VB45B: VH),

(9) SEQ ID NO: 140 (VB8B: VH),

(10) SEQ ID NO: 142 (VB115: VH),

(11) SEQ ID NO: 144 (VB14B: VH),

(12) SEQ ID NO: 118 (VB22B: VH),

5   (13) SEQ ID NO: 146 (VB16: VH),

(14) SEQ ID NO: 148 (VB157: VH),

(15) SEQ ID NO: 150 (VB4B: VH),

(16) SEQ ID NO: 152 (VB51: VH),

(17) SEQ ID NO: 155 (AB317: VH),

10   (18) SEQ ID NO: 159 (AB324: VH),

(19) SEQ ID NO: 162 (TA136: VH),

(20) SEQ ID NO: 229 (hVB22B p-z: VH),

(21) SEQ ID NO: 256 (hVB22B g-e: VH),

(22) SEQ ID NO: 262 (hVB22B e: VH),

15   (23) SEQ ID NO: 289 (hVB22B u2-wz4: VH),

(24) SEQ ID NO: 295 (hVB22B q-wz5: VH).

(IV) Antibody comprising a VL that comprises an amino acid sequence
of the SEQ ID NO in any one of (1) to (18):

20   (1) SEQ ID NO: 125 (VA7: VL),

(2) SEQ ID NO: 127 (VA130, VB17B, VB12B, VB115 or VB14B: VL),

(3) SEQ ID NO: 129 (VA259: VL),

(4) SEQ ID NO: 135 (VB140 or VB45B: VL),

(5) SEQ ID NO: 137 (VB33: VL),

25   (6) SEQ ID NO: 141 (VB8B: VL),

(7) SEQ ID NO: 120 (VB22B: VL),

(8) SEQ ID NO: 147 (VB16: VL),

(9) SEQ ID NO: 149 (VB157: VL),

(10) SEQ ID NO: 151 (VB4B: VL),

30   (11) SEQ ID NO: 153 (VB51: VL),

(12) SEQ ID NO: 157 (AB317: VL),

(13) SEQ ID NO: 161 (AB324: VL),

(14) SEQ ID NO: 163 (TA136: VL),

(15) SEQ ID NO: 238 (hVB22B p-z: VL),

35   (16) SEQ ID NO: 258 (hVB22B g-e: VL or hVB22B e: VL),

(17) SEQ ID NO: 291 (hVB22B u2-wz4: VL),

(18) SEQ ID NO: 297 (hVB22B q-wz5: VL).

(V) Antibody comprising a VH and VL according to any one of (1) to
(18):

5 (1) SEQ ID NOs: 3, 4, and 5 (VA7: H chain CDR1, 2, and 3); SEQ ID
NOs: 60, 61, and 62 (VA7: L chain CDR1, 2, and 3),
(2) SEQ ID NOs: 6, 7, and 8 (VA130 or VB17B: H chain CDR1, 2, and
3), SEQ ID NOs: 63, 64, and 65 (VA130 or VB17B: L chain CDR1, 2, and
3),
10 (3) SEQ ID NOs: 9, 10, and 11 (VA259: H chain CDR1, 2, and 3); SEQ
ID NOs: 66, 67, and 68 (VA259: L chain CDR1, 2, and 3),
(4) SEQ ID NOs: 15, 16, and 17 (VB12B: H chain CDR1, 2, and 3); SEQ
ID NO: 72, 73, and 74 (VB12B: L chain CDR1, 2, and 3),
(5) SEQ ID NOs: 18, 19, and 20 (VB140: H chain CDR1, 2, and 3); SEQ
15 ID NOs: 75, 76, and 77 (VB140: L chain CDR1, 2, and 3),
(6) SEQ ID NOs: 21, 22, and 23 (VB33: H chain CDR1, 2, and 3); SEQ
ID NOs: 78, 79, and 80 (VB33: L chain CDR1, 2, and 3),
(7) SEQ ID NOs: 24, 25, and 26 (VB45B: H chain CDR1, 2, and 3); SEQ
ID NOs: 81, 82, and 83 (VB45B: L chain CDR1, 2, and 3),
20 (8) SEQ ID NOs: 27, 28, and 29 (VB8B: H chain CDR1, 2, and 3); SEQ
ID NOs: 84, 85, and 86 (VB8B: L chain CDR1, 2, and 3),
(9) SEQ ID NOs: 30, 31, and 32 (VB115: H chain CDR1, 2, and 3); SEQ
ID NOs: 87, 88, and 89 (VB115: L chain CDR1, 2, and 3),
(10) SEQ ID NOs: 33, 34, and 35 (VB14B: H chain CDR1, 2, and 3); SEQ
25 ID NOs: 90, 91, and 92 (VB14B: L chain CDR1, 2, and 3),
(11) SEQ ID NOs: 36, 37, and 38 (VB22B, hVB22B p-z, hVB22B g-e, hVB22B
e, hVB22B u2-wz4 or hVB22B q-wz5: H chain CDR1, 2, and 3); SEQ ID
NOs: 93, 94, and 95 (VB22B, hVB22B p-z, hVB22B g-e, hVB22B e, hVB22B
u2-wz4 or hVB22B q-wz5: L chain CDR1, 2, and 3),
30 (12) SEQ ID NOs: 39, 40, and 41 (VB16: H chain CDR1, 2, and 3); SEQ
ID NOs: 96, 97, and 98 (VB16: L chain CDR1, 2, and 3),
(13) SEQ ID NOs: 42, 43, and 44 (VB157: H chain CDR1, 2, and 3); SEQ
ID NOs: 99, 100, and 101 (VB157: L chain CDR1, 2, and 3),
(14) SEQ ID NOs: 45, 46, and 47 (VB4B: H chain CDR1, 2, and 3); SEQ
35 ID NOs: 102, 103, and 104 (VB4B: L chain CDR1, 2, and 3),
(15) SEQ ID NOs: 48, 49, and 50 (VB51: H chain CDR1, 2, and 3); SEQ

ID NOs: 105, 106, and 107 (VB51: L chain CDR1, 2, and 3),

(16) SEQ ID NOs: 51, 52, and 53 (AB317: H chain CDR1, 2, and 3); SEQ ID NOs: 108, 109, and 110 (AB317: L chain CDR1, 2, and 3),

(17) SEQ ID NOs: 54, 55, and 56 (AB324: H chain CDR1, 2, and 3); SEQ

5 ID NOs: 111, 112, and 113 (AB324: L chain CDR1, 2, and 3),

(18) SEQ ID NOs: 57, 58, and 59 (TA136: H chain CDR1, 2, and 3); SEQ ID NOs: 114, 115, and 116 (TA136: L chain CDR1, 2, and 3).

(VI) Antibody comprising a VH and a VL that comprise the amino acid

10 sequences according to SEQ ID NOs in any one of (1) to (24) indicated below:

(1) SEQ ID NO: 124 (VA7: VH), SEQ ID NO: 125 (VA7: VL),

(2) SEQ ID NO: 126 (VA130: VH), SEQ ID NO: 127 (VA130: VL),

(3) SEQ ID NO: 128 (VA259: VH), SEQ ID NO: 129 (VA259: VL),

15 (4) SEQ ID NO: 130 (VB17B: VH), SEQ ID NO: 127 (VB17B: VL),

(5) SEQ ID NO: 132 (VB12B: VH), SEQ ID NO: 127 (VB12B: VL),

(6) SEQ ID NO: 134 (VB140: VH), SEQ ID NO: 135 (VB140: VL),

(7) SEQ ID NO: 136 (VB33: VH), SEQ ID NO: 137 (VB33: VL),

(8) SEQ ID NO: 138 (VB45B: VH), SEQ ID NO: 135 (VB45B: VL),

20 (9) SEQ ID NO: 140 (VB8B: VH), SEQ ID NO: 141 (VB8B: VL),

(10) SEQ ID NO: 142 (VB115: VH), SEQ ID NO: 127 (VB115: VL),

(11) SEQ ID NO: 144 (VB14B: VH), SEQ ID NO: 127 (VB14B: VL),

(12) SEQ ID NO: 118 (VB22B: VH), SEQ ID NO: 120 (VB22B: VL),

(13) SEQ ID NO: 146 (VB16: VH), SEQ ID NO: 147 (VB16: VL),

25 (14) SEQ ID NO: 148 (VB157: VH), SEQ ID NO: 149 (VB157: VL),

(15) SEQ ID NO: 150 (VB4B: VH), SEQ ID NO: 151 (VB4B: VL),

(16) SEQ ID NO: 152 (VB51: VH), SEQ ID NO: 153 (VB51: VL),

(17) SEQ ID NO: 155 (AB317: VH), SEQ ID NO: 157 (AB317: VL),

(18) SEQ ID NO: 159 (AB324: VH), SEQ ID NO: 161 (AB324: VL),

30 (19) SEQ ID NO: 162 (TA136: VH), SEQ ID NO: 163 (TA136: VL),

(20) SEQ ID NO: 229 (hVB22B p-z: VH), SEQ ID NO: 238 (hVB22B p-z: VL),

(21) SEQ ID NO: 256 (hVB22B g-e: VH), SEQ ID NO: 258 (hVB22B g-e: VL),

35 (22) SEQ ID NO: 262 (hVB22B e: VH), SEQ ID NO: 258 (hVB22B e: VL),

(23) SEQ ID NO: 289 (hVB22B u2-wz4: VH), SEQ ID NO: 291 (hVB22B u2-wz4:

VL),

(24) SEQ ID NO: 295 (hVB22B q-wz5: VH), SEQ ID NO: 297 (hVB22B q-wz5: VL).

5   (VII) Antibody comprising the amino acid sequence of SEQ ID NO: 122 (VB22B: scFv).

(VIII)   Humanized antibody comprising an amino acid sequence according to any one of SEQ ID NO: 2 (hVB22B p-z: sc(Fv)2), SEQ ID
10   NO: 254 (hVB22B g-e: sc(Fv)2), SEQ ID NO: 260 (hVB22B e: sc(Fv)2), SEQ ID NO: 287 (hVB22B u2-wz4: sc(Fv)2), and SEQ ID NO: 293 (hVB22B q-wz5: sc(Fv)2).

(IX) Antibody comprising a VH which has FR1, 2, 3, and 4 comprising
15   amino acid sequences according to SEQ ID NOs in any one of (1) to (5) indicated below:
(1) SEQ ID NOs: 230, 232, 234, and 236 (hVB22B p-z: H chain FR1, 2, 3, and 4),
(2) SEQ ID NOs: 265, 267, 269, and 271 (hVB22B g-e: H chain FR1, 2,
20   3, and 4),
(3) SEQ ID NOs: 279, 281, 283, and 285 (hVB22B e: H chain FR1, 2, 3, and 4),
(4) SEQ ID NOs: 298, 299, 300, and 301 (hVB22B u2-wz4: H chain FR1, 2, 3, and 4),
25   (5) SEQ ID NOs: 298, 299, 306, and 301 (hVB22B q-wz5: H chain FR1, 2, 3, and 4).

(X) Antibody comprising a VL which has FR1, 2, 3 and 4 comprising amino acid sequences according to SEQ ID NOs in any one of (1) to
30   (4) indicated below:
(1) SEQ ID NOs: 239, 241, 243, and 245 (hVB22B p-z: L chain FR1, 2, 3, and 4),
(2) SEQ ID NOs: 272, 274, 276, and 278 (hVB22B g-e or hVB22B e: L chain FR1, 2, 3, and 4),
35   (3) SEQ ID NOs: 302, 303, 304, and 305 (hVB22B u2-wz4: L chain FR1, 2, 3, and 4),

(4) SEQ ID NOs: 302, 307, 308, and 305 (hVB22B q-wz5: L chain FR1, 2, 3, and 4).

(XI) Antibody comprising VH and VL according to any one of (1) to (5) indicated below:

(1) VH having FR1, 2, 3, and 4 comprising the amino acid sequences of SEQ ID NOs: 230, 232, 234, and 236, respectively, and VL having FR1, 2, 3, and 4 comprising the amino acid sequences of SEQ ID NOs: 239, 241, 243, and 245, respectively;

(2) VH having FR1, 2, 3, and 4 comprising the amino acid sequences of SEQ ID NOs: 265, 267, 269, and 271, respectively, and VL having FR1, 2, 3, and 4 comprising the amino acid sequences of SEQ ID NOs: 272, 274, 276, and 278, respectively;

(3) VH having FR1, 2, 3, and 4 comprising the amino acid sequences of SEQ ID NOs: 279, 281, 283, and 285, respectively, and VL having FR1, 2, 3, and 4 comprising the amino acid sequences of SEQ ID NOs: 272, 274, 276, and 278, respectively;

(4) VH having FR1, 2, 3, and 4 comprising the amino acid sequences of SEQ ID NOs: 298, 299, 300, and 301, respectively, and VL having FR1, 2, 3, and 4 comprising the amino acid sequences of SEQ ID NOs: 302, 303, 304, and 305, respectively;

(5) VH having FR1, 2, 3, and 4 comprising the amino acid sequences of SEQ ID NOs: 298, 299, 306, and 301, respectively, and VL having FR1, 2, 3, and 4 comprising the amino acid sequences of SEQ ID NOs: 302, 307, 308, and 305, respectively.

(XII) Antibody comprising the amino acid sequence of SEQ ID NO: 264 (VB22B: sc(Fv)2).

An antibody comprising an amino acid sequence of any one of (I) to (XII) indicated above, in which one or more amino acids have been substituted, deleted, added, and/or inserted, wherein the antibody has activity equivalent to that of the antibody of any one of (I) to (XII).

Herein, the phrase "functionally equivalent" means that an antibody of interest has a biological or biochemical activity comparable to that of an antibody of the present invention. Such

activities include, for example, binding activities and agonistic activities.

Methods for preparing polypeptides functionally equivalent to a certain polypeptide are well known to those skilled in the art,

5 and include methods of introducing mutations into polypeptides. For example, those skilled in the art can prepare an antibody functionally equivalent to the antibodies of the present invention by introducing appropriate mutations into the antibody using site-directed mutagenesis (Hashimoto-Gotoh, T. *et al.* Gene 152, 271-275, (1995);

10 Zoller, MJ, and Smith, M. Methods Enzymol. 100, 468-500, (1983); Kramer, W. *et al.*, Nucleic Acids Res. 12, 9441-9456, (1984); Kramer, W. and Fritz HJ, Methods Enzymol. 154, 350-367, (1987); Kunkel, TA, Proc. Natl. Acad. Sci. USA. 82, 488-492, (1985); Kunkel, Methods Enzymol. 85, 2763-2766, (1988)), or such. Amino acid mutations may

15 occur naturally. Thus, the present invention also comprises antibodies functionally equivalent to the antibodies of the present invention and comprising the amino acid sequences of these antibodies, in which one or more amino acids is mutated. In such mutants, the number of amino acids that may be mutated is not particularly

20 restricted, so long as the activity is maintained. Generally, the number of amino acids that are mutated is 50 amino acids or less, preferably 30 or less, more preferably 10 or less (for example, five amino acids or less). Likewise, the site of mutation is not particularly restricted, so long as the mutation does not result in

25 the disruption of activity.

Amino acid mutations may be made at one or more predicted, preferably nonessential, amino acid residues. A "nonessential" amino acid residue is a residue that can be altered from the wild-type sequence of a protein without altering the biological activity,

30 whereas an "essential" amino acid residue is required for biological activity. An amino acid is preferably substituted for a different amino acid(s) that allows the properties of the amino acid side-chain to be conserved. Accordingly, throughout the present application, a "conservative amino acid substitution" means a replacement of an

35 amino acid residue belonging to one of the following groups with another amino acid in the same group having a chemically similar side

chain. Groups of amino acid residues having similar side chains have been defined in the art. Examples of amino acid side chain properties are: hydrophobic amino acids (A, I, L, M, F, P, W, Y, and V), hydrophilic amino acids (R, D, N, C, E, Q, G, H, K, S, and T), amino acids comprising

5   the following side chains: aliphatic side chains (G, A, V, L, I, and P); hydroxyl-containing side chains (S, T, and Y); sulfur-containing side chains (C and M); carboxylic acid- and amide-containing side chains (D, N, E, and Q); basic side chains (R, K, and H); aromatic ring-containing side chains (H, F, Y, and W) (amino acids are

10   represented by one-letter codes in parentheses).

A polypeptide comprising a modified amino acid sequence, in which one or more amino acid residues is deleted, added, and/or replaced with other amino acids, is known to retain its original biological activity (Mark, D. F. *et al.*, Proc. Natl. Acad. Sci. USA

15   81, 5662-5666 (1984); Zoller, M. J. & Smith, M. Nucleic Acids Research 10, 6487-6500 (1982); Wang, A. *et al.*, Science 224, 1431-1433; Dalbadie-McFarland, G. *et al.*, Proc. Natl. Acad. Sci. USA 79, 6409-6413 (1982)).

Fusion proteins containing antibodies that comprise the amino

20   acid sequence of an antibody of the present invention, in which two or more amino acid residues have been added, are included in the present invention. The fusion protein results from a fusion between one of the above antibodies and a second peptide or protein, and is included in the present invention. The fusion protein can be prepared

25   by ligating a polynucleotide encoding an antibody of the present invention and a polynucleotide encoding a second peptide or polypeptide in frame, inserting this into an expression vector, and expressing the fusion construct in a host. Some techniques known to those skilled in the art are available for this purpose. The partner

30   peptide or polypeptide to be fused with an antibody of the present invention may be a known peptide, for example, FLAG (Hopp, T. P. *et al.*, BioTechnology 6, 1204-1210 (1988)), 6x His consisting of six His (histidine) residues, 10x His, influenza hemagglutinin (HA), human c-myc fragment, VSV-GP fragment, p18HIV fragment, T7-tag,

35   HSV-tag, E-tag, SV40 T antigen fragment, lck tag, α-tubulin fragment, B-tag, Protein C fragment. Other partner polypeptides to be fused

with the antibodies of the present invention include, for example, GST (glutathione-S-transferase), HA (influenza hemagglutinin), immunoglobulin constant region, β-galactosidase, and MBP (maltose-binding protein). A polynucleotide encoding one of these 5 commercially available peptides or polypeptides can be fused with a polynucleotide encoding an antibody of the present invention. The fusion polypeptide can be prepared by expressing the fusion construct.

As described below, the antibodies of the present invention may differ in amino acid sequence, molecular weight, isoelectric point, 10 presence/absence of sugar chains, and conformation depending on the cell or host producing the antibody, or purification method. However, a resulting antibody is included in the present invention, as long as it is functionally equivalent to an antibody of the present invention. For example, when an antibody of the present invention 15 is expressed in prokaryotic cells, for example *E. coli*, a methionine residue is added to the N terminus of the original antibody amino acid sequence. Such antibodies are included in the present invention.

An antibody that recognizes an epitope recognized by the 20 antibody according to any one of (I) to (XII) indicated above is expected to have a high agonistic activity. Such antibodies can be prepared by methods known to those skilled in the art. The antibody can be prepared by, for example, determining the epitope recognized by the antibody according to any one of (I) to (XII) by conventional 25 methods, and using a polypeptide comprising one of the epitope amino acid sequences as an immunogen. Alternatively, the antibody can be prepared by determining the epitopes of conventionally prepared antibodies and selecting an antibody that recognizes the epitope recognized by an antibody of any one of (I) to (XII).

30 In the present invention, a particularly preferred antibody is an antibody that recognizes the epitope recognized by the antibody comprising the amino acid sequence of SEQ ID NO: 2. The antibody comprising the amino acid sequence of SEQ ID NO: 2 is predicted to recognize the region from Glu 26 to Leu 274, preferably the region 35 from Ala 189 to Gly 245, more preferably the region from Gln 213 to Ala 231 of human Mpl. Thus, antibodies recognizing the region of amino

acids 26 to 274, or amino acids 189 to 245, or amino acids 213 to 231 of human Mpl are also included in the present invention.

Antibodies recognizing regions of amino acids 26 to 274, amino acids 189 to 245, or amino acids 213 to 231 of the human Mpl amino acid sequence (SEQ ID NO: 123) can be obtained by methods known to those skilled in the art. Such antibodies can be prepared by, for example, using a peptide comprising amino acids 26 to 274, amino acids 189 to 245, or amino acids 213 to 231 of the human Mpl amino acid sequence (SEQ ID NO: 123) as an immunogen. Alternatively, such antibodies can be prepared by determining the epitope of a conventionally prepared antibody and selecting an antibody that recognizes the same epitope recognized by an antibody of the present invention.

The present invention provides antibodies described above in (I) to (XII). In an embodiment of the present invention, a preferred antibody is the one shown in (V), a more preferred antibody is the one shown in (VI), and a still more preferred is the one shown in (VIII).

The present invention also provides vectors comprising polynucleotides encoding the antibodies of the present invention, or polynucleotides which hybridize under stringent conditions to the polynucleotides of the present invention and encode antibodies having activities equivalent to those of the antibodies of the present invention. The polynucleotides of the present invention are polymers comprising multiple bases or base pairs of deoxyribonucleic acids (DNA) or ribonucleic acids (RNA), and are not particularly limited, as long as they encode the antibodies of the present invention. They may also contain non-natural nucleotides. The polynucleotides of the present invention can be used to express antibodies using genetic engineering techniques. The polynucleotides of this invention can also be used as probes in the screening of antibodies functionally equivalent to the antibodies of the present invention. Specifically, DNAs that hybridize under stringent conditions to a polynucleotide encoding an antibody of the present invention, and encode antibodies having activity equivalent to those of the antibodies of the present invention can be obtained by techniques such as hybridization and

gene amplification (for example, PCR), using a polynucleotide of the present invention or a portion thereof as a probe. Such DNAs are also included in the polynucleotides of the present invention. Hybridization techniques are well known to those skilled in the art (Sambrook, J et al., Molecular Cloning 2nd ed., 9.47-9.58, Cold Spring Harbor Lab. press, 1989). Such hybridization conditions include, for example, conditions of low stringency. Examples of conditions of low stringency include post-hybridization washing in 0.1x SSC and 0.1% SDS at 42°C, and preferably in 0.1x SSC and 0.1% SDS at 50°C. More preferable hybridization conditions include those of high stringency. Highly stringent conditions include, for example, washing in 5x SSC and 0.1% SDS at 65°C. In these conditions, the higher the temperature, the higher the expectation of efficiently obtaining polynucleotides with a high homology. However, several factors, such as temperature and salt concentration, can influence hybridization stringency, and those skilled in the art can suitably select these factors to accomplish similar stringencies.

Antibodies that are encoded by polynucleotides obtained by the hybridization and gene amplification techniques, and are functionally equivalent to the antibodies of the present invention generally exhibit high homology to the antibodies of the this invention at the amino acid level. The antibodies of the present invention include antibodies that are functionally equivalent to the antibodies of the present invention, and exhibit high amino acid sequence homology to the antibodies of this invention. The term "high homology" generally means identity at the amino acid level of at least 50% or higher, preferably 75% or higher, more preferably 85% or higher, still more preferably 95% or higher. Polypeptide homology can be determined by the algorithm described in the report: Wilbur, W. J. and Lipman, D. J. Proc. Natl. Acad. Sci. USA 80, 726-730 (1983).

When E. coli is used as a host, there is no particular limitation as to the type of vector of the present invention, as long as the vector contains an "ori" responsible for its replication in E. coli and a marker gene. The "ori" ensures the amplification and mass production of the vector in E. coli (for example, JM109, DH5α, HB101, and XL1Blue). The marker gene is used to select the E. coli

transformants (for example, a drug resistance gene selected by an appropriate drug such as ampicillin, tetracycline, kanamycin, and chloramphenicol). The vectors include, for example, M13 vectors, pUC vectors, pBR322, pBluescript, and pCR-Script. In addition to the

5   above vectors, for example, pGEM-T, pDIRECT, and pT7 can also be used for the subcloning and excision of cDNAs.

In particular, expression vectors are useful as vectors of the present invention. When an expression vector is expressed, for example, in *E. coli*, it should have the above characteristics in order

10 to be amplified in *E. coli*. Additionally, when *E. coli*, such as JM109, DH5α, HB101, or XL1-Blue are used as the host cell, the vector preferably has a promoter, for example, lacZ promoter (Ward *et al.* (1989) Nature 341:544-546; (1992) FASEB J. 6:2422-2427), araB promoter (Better *et al.* (1988) Science 240:1041-1043), or T7 promoter,

15 that allows efficient expression of the desired gene in *E. coli*. Other examples of the vectors include pGEX-5X-1 (Pharmacia), "QIAexpress system" (QIAGEN), pEGFP, and pET (where BL21, a strain expressing T7 RNA polymerase, is preferably used as the host).

Furthermore, the vectors may comprise a signal sequence for

20 polypeptide secretion. When producing polypeptides into the periplasm of *E. coli*, the pelB signal sequence (Lei, S. P. *et al.* J. Bacteriol. 169:4379 (1987)) may be used as a signal sequence for polypeptide secretion. For example, calcium chloride methods or electroporation methods may be used to introduce the vector into a

25 host cell.

In addition to *E. coli*, expression vectors derived from mammals (e.g., pCDNA3 (Invitrogen), pEGF-BOS (Nucleic Acids Res. (1990) 18(17):5322), pEF, pCDM8), insect cells (e.g., "Bac-to-BAC baculovirus expression system" (GIBCO-BRL), pBacPAK8), plants (e.g.,

30 pMH1, pMH2), animal viruses (e.g., pHSV, pMV, pAdexLcw), retroviruses (e.g., pZIPneo), yeasts (e.g., "Pichia Expression Kit" (Invitrogen), pNV11, SP-Q01), and *Bacillus subtilis* (e.g., pPL608, pKTH50) may also be used as a vector of the present invention.

In order to express proteins in animal cells such as CHO, COS,

35 and NIH3T3 cells, the vector preferably has a promoter necessary for expression in such cells, for example, an SV40 promoter (Mulligan

et al. (1979) Nature 277:108), MMLV-LTR promoter, EF1α promoter
(Mizushima *et al.* (1990) Nucleic Acids Res. 18:5322), CMV promoter,
etc.). It is even more preferable that the vector also carries a
marker gene for selecting transformants (for example, a
5   drug-resistance gene selected by a drug such as neomycin and G418.
Examples of vectors with such characteristics include pMAM, pDR2,
pBK-RSV, pBK-CMV, pOPRSV, and pOP13, and such.

In addition, to stably express a gene and amplify the gene copy
number in cells, CHO cells that are defective in the nucleic acid
10   synthesis pathway are introduced with a vector containing a DHFR gene
(for example, pCHOI) to compensate for the defect, and the copy number
is amplified using methotrexate (MTX). Alternatively, a COS cell,
which carries an SV40 T antigen-expressing gene on its chromosome,
can be transformed with a vector containing the SV40 replication
15   origin (for example, pcD) for transient gene expression. The
replication origin may be derived from polyoma virus, adenovirus,
bovine papilloma virus (BPV), and such. Furthermore, to increase the
gene copy number in host cells, the expression vector may contain,
as a selection marker, aminoglycoside transferase (APH) gene,
20   thymidine kinase (TK) gene, *E. coli* xanthine guanine phosphoribosyl
transferase (Ecogpt) gene, dihydrofolate reductase (dhfr) gene, and
such.

In the present invention, next, the vector is introduced into
a host cell. The host cells into which the vector is introduced are
25   not particularly limited, for example, *E. coli* and various animal
cells are available for this purpose. The host cells may be used,
for example, as a production system to produce and express the
antibodies of the present invention. *In vitro* and *in vivo* production
systems are available for polypeptide production systems.
30   Production systems that use eukaryotic cells or prokaryotic cells
are examples of *in vitro* production systems.

Eukaryotic cells that can be used are, for example, animal cells,
plant cells, and fungal cells. Known animal cells include: mammalian
cells, for example, CHO (J. Exp. Med. (1995)108, 945), COS, 3T3,
35   myeloma, BHK (baby hamster kidney), HeLa, Vero, amphibian cells such
as *Xenopus laevis* oocytes (Valle, *et al.* (1981) Nature 291, 358-340),

or insect cells (e.g., Sf9, Sf21, and Tn5). In the present invention, CHO-DG44, CHO-DXB11, COS7 cells, and BHK cells can be suitably used. Among animal cells, CHO cells are particularly favorable for large-scale expression. Vectors can be introduced into a host cell by, for example, calcium phosphate methods, the DEAE-dextran methods, methods using cationic liposome DOTAP (Boehringer-Mannheim), electroporation methods, lipofection methods.

Plant cells include, for example, Nicotiana tabacum-derived cells known as a protein production system. Calluses may be cultured from these cells. Known fungal cells include yeast cells, for example, genus Saccharomyces such as Saccharomyces cerevisiae and Saccharomyces pombe; and filamentous fungi, for example, genus Aspergillus such as Aspergillus niger.

Bacterial cells can be used in the prokaryotic production systems. Examples of bacterial cells include E. coli (for example, JM109, DH5α, HB101 and such); and Bacillus subtilis.

Next, the above host cells are cultured. Antibodies can be obtained by transforming the cells with a polynucleotide of interest and in vitro culturing of these transformants. Transformants can be cultured using known methods. For example, DMEM, MEM, RPMI 1640, or IMDM may be used as the culture medium for animal cells, and may be used with or without serum supplements such as FBS or fetal calf serum (FCS). Serum-free cultures are also acceptable. The preferred pH is about 6 to 8 during the course of culturing. Incubation is carried out typically at a temperature of about 30 to 40°C for about 15 to 200 hours. Medium is exchanged, aerated, or agitated, as necessary.

On the other hand, production systems using animal or plant hosts may be used as systems for producing polypeptides in vivo. For example, a polynucleotide of interest is introduced into an animal or plant and the polypeptide is produced in the body of the animal or plant and then recovered. The "hosts" of the present invention includes such animals and plants.

Animals to be used for the production system include mammals or insects. Mammals such as goats, pigs, sheep, mice, and cattle may be used (Vicki Glaser SPECTRUM Biotechnology Applications (1993)). Alternatively, the mammals may be transgenic animals.

For example, a polynucleotide of interest is prepared as a fusion gene with a gene encoding a polypeptide specifically produced in milk, such as the goat β-casein gene. DNA fragments containing the fusion gene are injected into goat embryos, which are then

5 introduced back to female goats. The desired antibody can be obtained from milk produced by the transgenic goats, which are born from the goats that received the embryos, or from their offspring. Appropriate hormones may be administered to increase the volume of milk containing the antibody produced by the transgenic goats (Ebert,

10 K.M. *et al.*, Bio/Technology 12, 699-702 (1994)).

Insects, such as silkworms, may also be used. Baculoviruses carrying a polynucleotide encoding an antibody of interest can be used to infect silkworms, and the antibody of interest can be obtained from the body fluids (Susumu, M. *et al.*, Nature 315, 592-594 (1985)).

15 Plants used in the production system include, for example, tobacco. When tobacco is used, a polynucleotide encoding an antibody of interest is inserted into a plant expression vector, for example, pMON 530, and then the vector is introduced into a bacterium, such as Agrobacterium tumefaciens. The bacteria are then used to infect

20 tobacco such as Nicotiana tabacum, and the desired antibodies can be recovered from the leaves (Julian K.-C. Ma *et al.*, Eur. J. Immunol. 24, 131-138 (1994)).

The resulting antibody may be isolated from the inside or outside (such as the medium) of host cells, and purified as a

25 substantially pure and homogenous antibody. Methods are not limited to any specific method and any standard method for isolating and purifying antibodies may be used. Polypeptides may be isolated and purified, by selecting an appropriate combination of, for example, chromatographic columns, filtration, ultrafiltration, salting out,

30 solvent precipitation, solvent extraction, distillation, immunoprecipitation, SDS-polyacrylamide gel electrophoresis, isoelectric focusing, dialysis, recrystallization, and others. The term "substantially pure" as used herein in reference to a given polypeptide means that the polypeptide is substantially free from

35 contaminants such as other biological macromolecules, culture media (if recombinantly produced), or chemical precursors (if chemically

synthesized). The substantially pure polypeptide is at least 75%, preferably at least about 80%, more preferably at least about 85, 90, 95, or 99% pure by dry weight. Purity can be measured by any appropriate standard method, for example by a chromatography method,

5 polyacrylamide gel electrophoresis, or HPLC analysis.

Chromatographies include, for example, affinity chromatographies, ion exchange chromatographies, hydrophobic chromatographies, gel filtrations, reverse-phase chromatographies, and adsorption chromatographies (Strategies for Protein Purification

10 and Characterization: A Laboratory Course Manual. Ed Daniel R. Marshak *et al.*, Cold Spring Harbor Laboratory Press, 1996). These chromatographies can be carried out using liquid phase chromatographies such as HPLC and FPLC. Examples of the affinity chromatography columns include protein A columns and protein G columns.

15 Examples of the proteins A columns include Hyper D, POROS, and Sepharose F. F. (Pharmacia).

An antibody can be modified freely and peptide portions deleted by treating the antibody with an appropriate protein modifying enzyme before or after antibody purification. Such protein modifying

20 enzymes include, for example, trypsins, chymotrypsins, lysyl endopeptidases, protein kinases, and glucosidases.

Antibodies that bind to Mpl can be prepared by methods known to those skilled in the art.

For example, monoclonal antibody-producing hybridomas can be

25 essentially generated by known technologies as follows: immunizing animals with Mpl proteins or Mpl-expressing cells as sensitized antigens using conventional immunological methods; fusing the obtained immunocytes with known parental cells by conventional cell fusion methods; and screening for monoclonal antibody-producing cells

30 by conventional methods.

Specifically, monoclonal antibodies can be prepared by the method below.

First, Mpl protein, which is used as a sensitized antigen for preparing antibodies, is prepared by expressing the Mpl gene/amino

35 acid sequence (GenBank accession number: NM_005373). More specifically, the gene sequence encoding Mpl is inserted into a known

expression vector, which is then transfected into an appropriate host cell. The subject human Mpl protein is purified from the host cell or culture supernatant using known methods.

The purified Mpl protein is then used as a sensitized antigen.
5   Alternatively, a partial Mpl peptide may be used as a sensitized antigen. In this case, the partial peptide can also be chemically synthesized based on the amino acid sequence of human Mpl.

The epitopes of Mpl molecule that are recognized by an anti-Mpl antibody of the present invention are not limited to a particular
10   epitope, and may be any epitope on the Mpl molecule. Thus, any fragment can be used as an antigen for preparing anti-Mpl antibodies of the present invention, as long as the fragment comprises an epitope of the Mpl molecule.

There is no limitation as to the type of mammalian species to
15   be immunized with the sensitized antigen. However, a mammal is preferably selected based on its compatibility with the parental cell to be used in cell fusion. Generally, rodents (for example, mice, rats, and hamsters), rabbits, and monkeys can be used.

Animals can be immunized with a sensitized antigen by known
20   methods such as a routine method of injecting a sensitized antigen into a mammal intraperitoneally or subcutaneously. Specifically, the sensitized antigen is diluted appropriately with phosphate-buffered saline (PBS), physiological saline and such, and then suspended. An adequate amount of a conventional adjuvant, for
25   example, Freund's complete adjuvant, is mixed with the suspension, as necessary. An emulsion is then prepared for administering to a mammal several times over a 4- to 21-day interval. An appropriate carrier may be used for the sensitized antigen in immunization.

A mammal is immunized as described above. After a titer
30   increase of target antibody in the serum is confirmed, immunocytes are collected from the mammal and then subjected to cell fusion. Spleen cells are the preferred immunocytes.

Mammalian myeloma cells are used as the parental cells to be fused with the above immunocytes. Preferable myeloma cells to be used
35   include various known cell lines, for example, P3 (P3x63Ag8.653) (Kearney JF, et al., J. Immnol. 123, 1548-1550 (1979)), P3x63Ag8U.1

(Yelton DE, *et al.*, Current Topics in Microbiology and Immunology 81, 1-7 (1978)), NS-1 (Kohler, G. and Milstein, C. Eur. J. Immunol. 6, 511-519 (1976)), MPC-11 (Margulies, D. H. *et al.*, Cell 8, 405-415 (1976)), SP2/0 (Shulman, M. *et al.*, Nature 276, 269-270 (1978)), FO

5   (deSt. Groth, S. F. *et al.*, J. Immunol. Methods 35, 1-21 (1980)), S194 (Trowbridge, I. S., J. Exp. Med. 148, 313-323 (1978)), and R210 (Galfre, G. *et al.*, Nature 277, 131-133 (1979)).

       Cell fusions between the immunocytes and the myeloma cells as described above can be essentially carried out using known methods,

10   for example, a method by Kohler and Milstein (Kohler, G. and Milstein, C., Methods Enzymol. 73, 3-46 (1981)).

       More specifically, the above-described cell fusions are carried out, for example, in a conventional culture medium in the presence of a cell fusion-promoting agent.   The fusion-promoting agents

15   include, for example, polyethylene glycol (PEG) and Sendai virus (HVJ). If required, an auxiliary substance such as dimethyl sulfoxide may also be added to improve fusion efficiency.

       The ratio of immunocytes to myeloma cells may be determined at one's own discretion, preferably, for example, one myeloma cell for

20   every one to ten immunocytes.   Culture media to be used for the above cell fusions include, for example, media that are suitable for the growth of the above myeloma cell lines, such as RPMI 1640 media and MEM media, and other conventional culture media used for this type of cell culture.   In addition, serum supplements such as fetal calf

25   serum (FCS) may also be used in combination.

       Cell fusion is carried out as follows.   As described above, predetermined amounts of immunocytes and myeloma cells are mixed well in the culture medium.   PEG solution (for example, mean molecular weight of about 1,000-6,000) pre-heated to 37°C is added to the cell

30   suspension typically at a concentration of 30% to 60% (w/v), and mixed to produce fused cells (hybridomas).   Then, an appropriate culture medium is successively added to the mixture, and the sample is centrifuged to remove supernatant.   This treatment is repeated several times to remove the unwanted cell fusion-promoting agent and

35   others that are unfavorable to hybridoma growth.

       Screening of the resulting hybridomas can be carried out by

culturing them in a conventional selective medium, for example, hypoxanthine, aminopterin, and thymidine (HAT) medium. Culturing in the above-descried HAT medium is continued for a period long enough (typically, for several days to several weeks) to kill cells (non-fused cells) other than the desired hybridomas. Then, hybridomas are screened for single-cell clones capable of producing the target antibody by conventional limiting dilution methods.

In addition to the method for preparing the above-descried hybridomas by immunizing non-human animals with antigens, preferred human antibodies having binding activity to Mpl can also be obtained by: sensitizing human lymphocytes with Mpl *in vitro*; and fusing the sensitized lymphocytes with human myeloma cells capable of dividing permanently (see, Examined Published Japanese Patent Application No. (JP-B) Hei 1-59878). Alternatively, it is possible to obtain human antibodies against Mpl from immortalized cells producing anti-Mpl antibodies. In this method, the cells producing anti-Mpl antibodies are prepared by administering Mpl as an antigen to transgenic animals comprising a repertoire of the entire human antibody genes (see, WO 94/25585, WO 93/12227, WO 92/03918, and WO 94/02602).

The monoclonal antibody-producing hybridomas thus prepared can be passaged in a conventional culture medium, and stored in liquid nitrogen over long periods of time.

Monoclonal antibodies can be prepared from the above-described hybridomas by, for example, a routine procedure of culturing the hybridomas and obtaining antibodies from the culture supernatants. Alternatively, monoclonal antibodies can be prepared by injecting the hybridomas into a compatible mammal; growing these hybridomas in the mammal; and obtaining antibodies from the mammal's ascites. The former method is suitable for preparing highly purified antibodies, while the latter is suitable for preparing antibodies on a large scale.

Recombinant antibodies can also be prepared by: cloning an antibody gene from a hybridoma; inserting the gene into an appropriate vector; introducing the vector into a host; and producing the antibodies by using genetic recombination techniques (see, for example, Vandamme, A. M. *et al.*, Eur. J. Biochem. 192, 767-775, (1990)).

Specifically, an mRNA encoding the variable (V) region of anti-Mpl antibody is isolated from hybridomas producing the anti-Mpl antibodies. For mRNA isolation, total RNAs are first prepared by conventional methods such as guanidine ultracentrifugation methods

5   (Chirgwin, J. M. *et al.*, Biochemistry 18, 5294-5299 (1979)), or acid guanidinium thiocyanate-phenol-chloroform (AGPC) methods (Chomczynski, P. *et al.*, Anal. Biochem. 162, 156-159 (1987)), and then the target mRNA is prepared using an mRNA Purification Kit (Pharmacia) and such. Alternatively, the mRNA can be directly

10   prepared using the QuickPrep mRNA Purification Kit (Pharmacia).

A cDNA of the antibody V region is synthesized from the resulting mRNA using reverse transcriptase. cDNA synthesis is carried out using the AMV Reverse Transcriptase First-strand cDNA Synthesis Kit (Seikagaku Co.), or such. Alternatively, cDNA can be synthesized and

15   amplified by the 5'-RACE method (Frohman, M. A. *et al.*, Proc. Natl. Acad. Sci. USA 85, 8998-9002 (1988); Belyavsky, A. *et al.*, Nucleic Acids Res. 17, 2919-2932 (1989)) using the 5'-Ampli FINDER RACE Kit (Clontech) and PCR.

Target DNA fragments are purified from the obtained PCR products

20   and then ligated with vector DNAs to prepare recombinant vectors. The vectors are introduced into *E. coli* and such, and colonies are selected for preparing the recombinant vector of interest. The target DNA nucleotide sequence is then confirmed by conventional methods such as the dideoxynucleotide chain termination method.

25   Once a DNA encoding the V region of target anti-Mpl antibody is obtained, the DNA is inserted into an expression vector which comprises a DNA encoding the constant region (C region) of a desired antibody.

The method for producing anti-Mpl antibodies to be used in the

30   present invention typically comprises the steps of: inserting an antibody gene into an expression vector, so that the gene is expressed under the regulation of expression regulatory regions, such as enhancer and promotor; and transforming host cells with the resulting vectors to express antibodies.

35   For expressing the antibody gene, polynucleotides encoding H chain and L chain, respectively, are inserted into separate expression

vectors and co-transfected into a host cell. Alternatively, polynucleotides encoding both H chain and L chain are inserted into a single expression vector and transfected into a host cell (see WO 94/11523).

5   The term "agonistic activity" refers to an activity to induce changes in some biological activities through signal transduction into cells and such, due to the binding of an antibody to a receptor antigen. The biological activities include, for example, proliferation-promoting activities, proliferation activities,

10   viability activities, differentiation-inducing activities, differentiation activities, transcriptional activities, membrane transport activities, binding activities, proteolytic activities, phosphorylation/dephosphorylation activities, oxidation/reduction activities, transfer activities, nucleolytic activities,

15   dehydration activities, cell death-inducing activities, and apoptosis-inducing activities, but is not limited thereto.

The term "agonistic activity against Mpl" typically refers to the activity of promoting the differentiation of megakaryocytes or their parental hemopoietic stem cells into platelets, or the activity

20   of stimulating platelet proliferation.

Agonistic activity can be assayed by methods known to those skilled in the art. The agonistic activity may be determined using the original activity or a different activity as an indicator.

For example, agonistic activity can be determined by a method

25   using cell growth as an indicator as described in Examples. More specifically, an antibody whose agonistic activity is to be determined is added to cells which proliferate in an agonist-dependent manner, followed by incubation of the cells. Then, a reagent such as WST-8 which shows a coloring reaction at specific wavelengths depending

30   on the viable cell count, is added to the culture and absorbance is measured. The agonistic activity can be determined using the measured absorbance as an indicator.

Cells that proliferate in an agonist-dependent manner can also be prepared by methods known to those skilled in the art. For example,

35   when the antigen is a receptor capable of transducing cell growth signals, cells expressing the receptor may be used. Alternatively,

when the antigen is a receptor that cannot transduce signals, a chimeric receptor consisting of the intracellular domain of a receptor that transduces cell growth signals and the extracellular domain of a receptor that does not transduce cell growth signals can be prepared

5   for cellular expression. Receptors that transduce cell growth signals include, for example, G-CSF receptors, mpl, neu, GM-CSF receptors, EPO receptors, c-kit, and FLT-3. Cells that can be used to express a receptor include, for example, BaF3, NFS60, FDCP-1, FDCP-2, CTLL-2, DA-1, and KT-3.

10   There is no limitation as to the type of detection indicators to be used for determining agonistic activity, as long as the indicator can monitor quantitative and/or qualitative changes. For example, it is possible to use cell-free assay indicators, cell-based assay indicators, tissue-based assay indicators, and *in vivo* assay

15   indicators. Indicators that can be used in cell-free assays include enzymatic reactions, quantitative and/or qualitative changes in proteins, DNAs, or RNAs. Such enzymatic reactions include, for example, amino acid transfers, sugar transfers, dehydrations, dehydrogenations, and substrate cleavages. Alternatively, protein

20   phosphorylations, dephosphorylations, dimerizations, multimerizations, hydrolyses, dissociations and such; DNA or RNA amplifications, cleavages, and extensions can be used as the indicator in cell-free assays. For example, protein phosphorylations downstream of a signal transduction pathway may be used as a detection

25   indicator. Alterations in cell phenotype, for example, quantitative and/or qualitative alterations in products, alterations in growth activity, alterations in cell number, morphological alterations, or alterations in cellular properties, can be used as the indicator in cell-based assays. The products include, for example, secretory

30   proteins, surface antigens, intracellular proteins, and mRNAs. The morphological alterations include, for example, alterations in dendrite formation and/or dendrite number, alteration in cell flatness, alteration in cell elongation/axial ratio, alterations in cell size, alterations in intracellular structure,

35   heterogeneity/homogeneity of cell populations, and alterations in cell density. Such morphological alterations can be observed under

a microscope. Cellular properties to be used as the indicator include anchor dependency, cytokine-dependent response, hormone dependency, drug resistance, cell motility, cell migration activity, pulsatory activity, and alteration in intracellular substances. Cell motility includes cell infiltration activity and cell migration activity. The alterations in intracellular substances include, for example, alterations in enzyme activity, mRNA levels, levels of intracellular signaling molecules such as Ca^{2+} and cAMP, and intracellular protein levels. When a cell membrane receptor is used, alterations in the cell proliferating activity induced by receptor stimulation can be used as the indicator. The indicators to be used in tissue-based assays include functional alterations adequate for the subject tissue. In *in vivo* assays, alterations in tissue weight, alterations in the blood system (for example, alterations in blood cell counts, protein contents, or enzyme activities), alterations in electrolyte levels, and alterations in the circulating system (for example, alterations in blood pressure or heart rate).

The methods for measuring such detection indices are not particularly limited. For example, absorbance, luminescence, color development, fluorescence, radioactivity, fluorescence polarization, surface plasmon resonance signal, time-resolved fluorescence, mass, absorption spectrum, light scattering, and fluorescence resonance energy transfer may be used. These measurement methods are known to those skilled in the art and may be selected appropriately depending on the purpose. For example, absorption spectra can be obtained by using a conventional photometer, plate reader, or such; luminescence can be measured with a luminometer or such; and fluorescence can be measured with a fluorometer or such. Mass can be determined with a mass spectrometer. Radioactivity can be determined with a device such as a gamma counter depending on the type of radiation. Fluorescence polarization can be measured with BEACON (TaKaRa). Surface plasmon resonance signals can be obtained with BIACORE. Time-resolved fluorescence, fluorescence resonance energy transfer, or such can be measured with ARVO or such. Furthermore, a flow cytometer can also be used for measuring. It is possible to use one of the above methods to measure two or more different types of

detection indices. A greater number of detection indices may also
be examined by using two or more measurement methods simultaneously
and/or consecutively. For example, fluorescence and fluorescence
resonance energy transfer can be measured at the same time with a
5 fluorometer.

The present invention also provides pharmaceutical
compositions comprising antibodies of this invention. The
pharmaceutical compositions comprising antibodies of the present
invention are useful for treating and/or preventing thrombocytopenia
10 and such. Time required for the platelet count to recover to the
normal level can be shortened by administering an antibody of the
present invention after donation of platelet components. The amount
of platelet components at the time of blood collection can be increased
by pre-administering an antibody of the present invention.

15 When used as pharmaceutical compositions, the antibodies of the
present invention can be formulated by methods known to those skilled
in the art. For example, the antibodies can be administered
parenterally by injection of a sterile solution or suspension in water
or other pharmaceutically acceptable solvents. For example, the
20 antibodies can be formulated by appropriately combining with
pharmaceutically-acceptable carriers or solvents, specifically,
sterile water or physiological saline, vegetable oils, emulsifiers,
suspending agents, surfactants, stabilizers, flavoring agents,
excipients, vehicles, preservatives, binding agents, and such, and
25 mixing at a unit dosage and form required by accepted pharmaceutical
implementations. In such formulations, the amount of the thus
obtained active ingredient should be within the required range.

A sterile composition to be injected can be formulated using
a vehicle such as distilled water used for injection, according to
30 standard protocols.

Aqueous solutions used for injections include, for example,
physiological saline and isotonic solutions comprising glucose or
other adjunctive agents such as D-sorbitol, D-mannose, D-mannitol,
and sodium chloride. They may also be combined with an appropriate
35 solubilizing agent such as alcohol, specifically, ethanol,
polyalcohol such as propylene glycol or polyethylene glycol, or

non-ionic detergent such as polysorbate 80™ or HCO-50, as necessary.

Oil solutions include sesame oils and soybean oils, and can be combined with solubilizing agents such as benzyl benzoate or benzyl alcohol. Injection solutions may also be formulated with buffers, for example, phosphate buffers or sodium acetate buffers; analgesics, for example, procaine hydrochloride; stabilizers, for example, benzyl alcohol or phenol; or anti-oxidants. The prepared injections are typically aliquoted into appropriate ampules.

The administration is preferably carried out parenterally, specifically, by injection, intranasal administration, intrapulmonary administration, percutaneous administration, or such. Injections include, for example, intravenous injections, intramuscular injections, intraperitoneal injections, and subcutaneous injections. The injection solutions can be also administered systemically or locally.

The administration methods can be selected properly according to the patient's age, condition, and such. The applied dose of a pharmaceutical composition comprising an antibody or polynucleotide encoding the antibody may be, for example, in the range of 0.0001 to 1,000 mg/kg body weight. Alternatively, the dosage may be, for example, in the range of 0.001 to 100,000 mg/kg body weight. However, the dosage is not restricted to the values described above. The dosage and administration methods depend on the patient's weight, age, and condition, and are appropriately selected by those skilled in the art.

Furthermore, the present invention relates to methods for inducing signals in Mpl-expressing cells by using the antibodies of the present invention. More specifically, the present invention relates methods for inducing signals in Mpl-expressing cells, in which the methods comprise the step of contacting the cells with the antibodies of the present invention.

All patents, published patent applications, and publications cited herein are incorporated by reference in their entirety.

Examples

The present invention is specifically illustrated below with

reference to Examples, but it is not to be construed as being limited thereto.

[Example 1]   Preparation of anti-human Mpl antibodies

5   1.1 Establishment of Mpl-expressing BaF3 cell lines

BaF3 cell lines expressing the full-length Mpl gene were established to obtain cell lines that proliferate in a TPO-dependent manner.

A full-length human Mpl cDNA (Palacios, R. et al., Cell, 41,

10   727-734 (1985)) (GenBank accession NO. NM_005373) was amplified by PCR.   The cDNA was cloned into a pCOS2 expression vector to construct pCOS2-hMplfull.   The expression vector pCOS2 was constructed by removing the DHFR gene expression region from pCHOI (Hirata, Y. et al., FEBS Letter, 356, 244-248 (1994)), where the expression region

15   of the neomycin resistance gene HEF-VH-gγ1 (Sato, K. et al., Mol Immunol., 31, 371-381 (1994)) is inserted.

The cynomolgus monkey Mpl cDNA (SEQ ID NO: 164) was cloned from total RNA extracted from the bone marrow cells of cynomolgus monkey, using a SMART RACE cDNA Amplification Kit (Clontech).   The resulting

20   cynomolgus monkey cDNA was inserted into pCOS2 to construct pCOS2-monkeyMplfull.

Then, the full-length mouse Mpl cDNA (GenBank accession NO. NM_010823) was amplified by PCR, and inserted into pCOS2 to construct pCOS2-mouseMplfull.

25   Each vector (20 μg) prepared as described above was mixed with BaF3 cells (1x 10^7 cells/mL) suspended in PBS in Gene Pulser cuvettes. This mixture was then pulsed at 0.33 kV and 950 μFD using a Gene Pulser II (Bio-Rad).   The BaF3 cells introduced with the above DNAs by electroporation were added to RPMI 1640 medium (Invitrogen)

30   containing 1 ng/mL mouse interleukin 3 (hereinafter abbreviated as mIL-3; Peprotech), 500 μg/mL Geneticin (Invitrogen), and 10% FBS (Invitrogen), and selected to establish a human Mpl-expressing BaF3 cell line (hereinafter abbreviated as "BaF3-human Mpl"), monkey Mpl-expressing BaF3 cell line (hereinafter abbreviated as BaF3-monkey

35   Mpl), and mouse Mpl-expressing BaF3 cell line (hereinafter abbreviated as "BaF3-mouse Mpl").   Following selection, these cells

were cultured and maintained in RPMI 1640 containing 1 ng/mL rhTPO (R&D) and 10% FBS.

1.2 Establishment of Mpl-expressing CHO cell lines

CHO cell lines expressing the full-length Mpl gene were established to obtain cell lines to be used for assessing binding activity by flow cytometry.

First, the DHFR gene expression site from pCHOI was inserted into pCXN2 (Niwa, H. et al., Gene, 108, 193-199 (1991)) at the HindIII site to prepare a pCXND3expression vector. The respective Mpl genes were amplified by PCR using pCOS2-hMplfull, pCOS2-monkeyMplfull, and pCOS2-mouseMplfull as templates, and primers with a His-tag sequence. The PCR products were cloned into pCXND3 to construct pCXND3-hMpl-His, pCXND3-monkey Mpl-His, and pCXND3-mouse Mpl-His, respectively.

Vectors thus prepared (25 µg each) were mixed with a PBS suspension of CHO-DG44 cells (1×10^7 cells/mL) in Gene Pulser cuvettes. The mixture was then pulsed at 1.5 kV and 25 µFD using Gene Pulser II (Bio-Rad). The CHO cells introduced with these DNAs by electroporation were added to CHO-S-SFMII medium (Invitrogen) containing 500 µg/mL Geneticin and 1x HT (Invitrogen). A human Mpl-expressing CHO cell line (hereinafter abbreviated as "CHO-human Mpl"), monkey Mpl-expressing CHO cell line (hereinafter abbreviated as "CHO-monkey Mpl"), and mouse Mpl-expressing CHO cell line (hereinafter abbreviated as "CHO-mouse Mpl") were established through selection.

1.3 Preparation of soluble human Mpl protein

To prepare soluble human Mpl protein, an expression system using insect Sf9 cells for production and secretion of the protein was constructed as described below.

A DNA construct encoding the extracellular region of human Mpl (Gln 26 to Trp 491) with a downstream FLAG tag was prepared. The construct was inserted into a pBACSurf-1 Transfer Plasmid (Novagen) between the PstI and SmaI sites to prepare pBACSurf1-hMpl-FLAG. Then, Sf9 cells were transformed with 4 µg of pBACSurf1-hMpl-FLAG using the Bac-N-Blue Transfection Kit (Invitrogen). The culture

supernatant was collected after a three-day incubation.   Recombinant virus was isolated by plaque assays.   The prepared virus stock was used to infect Sf9 cells, and the culture supernatant was collected.

5   Soluble human Mpl protein was purified from the obtained culture supernatant as described below.   The culture supernatant was loaded onto a Q Sepharose Fast Flow (Amersham Biosciences) for adsorption, and the adsorbed protein was then eluted with 50 mM Na-phosphate buffer (pH7.2) containing 0.01% (v/v) Tween 20 and 500 mM NaCl.   After the eluates were loaded onto a FLAG M2-Agarose (Sigma-Aldrich) for

10   adsorption, the protein adsorbed was eluted with 100 mM glycine-HCl buffer (pH3.5) containing 0.01% (v/v) Tween 20.   Immediately after elution, the fraction obtained was neutralized with 1 M Tris-HCl Buffer (pH8.0) and the buffer was exchanged with PBS(-) and 0.01% (v/v) Tween 20 using PD-10 columns (Amersham Biosciences).   The

15   purified soluble Mpl protein was referred to as "shMpl-FLAG".

1.4 Preparation of human Mpl-IgG Fc fusion protein

      Human fusion protein Mpl-IgG Fc gene was prepared according to the method by Bennett *et al.* (Bennett, B. D. *et al.*, J. Biol. Chem.

20   266, 23060-23067 (1991)).   A nucleotide sequence encoding the extracellular region of human Mpl (Gln 26 to Trp 491) was linked to a nucleotide sequence encoding the Fc region of human IgG-γ1 (a region downstream of Asp 216).   A BstEII sequence (amino acids: Val-Thr) was attached to the junction as a fusion linker between these two regions.

25   A 19-amino acid signal peptide derived form human IgG H chain variable region was used as the signal sequence.   The resulting human fusion protein Mpl-IgG Fc gene was cloned into pCXND3 to construct pCXND3-hMpl-Fc.

      The vector thus prepared (25 μg) was mixed with a PBS suspension

30   of CHO-DG44 cells (1x 10^7 cells/mL) in Gene Pulser cuvettes.   The mixture was then pulsed at 1.5 kV and 25 μFD using Gene Pulser II (Bio-Rad).   The CHO cells introduced with the DNA by electroporation were added to CHO-S-SFMII medium containing 500 μg/mL Geneticin and 1x HT (Invitrogen).   shMPL-Fc-expressing CHO cell line (CHO-hMpl-Fc)

35   was then established through selection.

      Human Mpl-IgG Fc fusion protein was purified from the culture

supernatant as described below.

The culture supernatant was loaded onto a Q Sepharose Fast Flow (Amersham Biosciences) for adsorption, and then the adsorbed protein were eluted with 50 mM Na-phosphate buffer (pH7.6) containing 0.01% (v/v) Tween 20 and 1 M NaCl. After the eluates were loaded onto a HiTrap protein G HP column (Amersham Biosciences) for adsorption, the adsorbed protein was eluted with 0.1 M glycine-HCl buffer (pH2.7) containing 150 mM NaCl and 0.01% (v/v) Tween 20. Immediately after elution, the obtained fraction was neutralized with 1 M Tris-HCl Buffer (pH8.0) and the buffer was exchanged with PBS(-) and 0.01% (v/v) Tween 20 using PD-10 columns (Amersham Biosciences). The purified soluble Mpl protein was referred to as "hMpl-Fc".

1.5 Immunization with shMpl-FLAG or BaF3-human Mpl and hybridoma selection

MRL/MpJUmmCrj-lpr/lpr mice (hereinafter abbreviated as "MRL/lpr mice"; purchased from Charles River, Japan) were immunized; the primary immunization was carried out at eight weeks of age. For every single mouse, an emulsion containing 100 μg of shMPL-FLAG combined with Freund's complete adjuvant (H37 Ra; Beckton Dickinson), was administered subcutaneously as the primary injection. As a booster injection, an emulsion containing shMPL-FLAG (50 μg per mouse) combined with Freund's incomplete adjuvant (Beckton Dickinson) was administered subcutaneously. Three mice which have been immunized six times in total were subjected to a final injection of shMPL-FLAG (50 μg per mouse) through the caudal vein. Cell fusion was achieved by mixing the mouse myeloma P3-X63Ag8U1 cells (P3U1; purchased from ATCC) and mouse splenocytes using polyethylene glycol 1500 (Roche Diagnostics). Hybridoma selection in HAT medium began the following day and culture supernatants were obtained. Screening was carried out by ELISA, using immunoplates immobilized with shMpl-FLAG or hMpl-Fc and the assayed cell growth activity of BaF3-human Mpl as an index. In addition, Balb/C mice were immunized eleven times in total by administering BaF3-human Mpl (1.0×10^7 cells per mouse) intraperitoneally over a period of one week to five months. Hybridomas were similarly prepared by cell fusion, and screened using

the assayed cell growth activity of BaF3-human Mpl as an index. Positive clones were isolated as single clones by limiting dilution and then cultured in a large scale. The culture supernatants were collected.

5

1.6 Analyses of anti-human Mpl antibodies

Antibody concentrations were determined by carrying out a mouse IgG sandwich ELISA using goat anti-mouse IgG (gamma) (ZYMED) and alkaline phosphatase-goat anti-mouse IgG (gamma) (ZYMED), generating 10 a calibration curve by GraphPad Prism (GraphPad Software; USA), and calculating the antibody concentrations from the calibration curve. Commercially available antibodies of the same isotype were used as standards.

Antibody isotypes were determined by antigen-dependent ELISA 15 using isotype-specific secondary antibodies. hMpl-Fc was diluted to 1 μg/mL with a coating buffer (0.1 mM $NaHCO_3$, pH9.6) containing 0.02% (w/v) NaN_3, and then added to ELISA plates. The plates were incubated overnight at 4°C for coating. The plates were blocked with a diluent buffer (50 mM Tris-HCl (pH8.1) containing 1 mM $MgCl_2$, 150 mM NaCl, 20 0.05% (v/v) Tween 20, 0.02% (w/v) NaN_3, 1% (w/v) BSA). After the addition of hybridoma culture supernatants, the plates were allowed to stand at room temperature for 1 hr. After washing with a rinse buffer (0.05% (v/v) Tween 20 in PBS), alkaline phosphatase-labeled isotype-specific secondary antibodies were added to the plates. Then, 25 the plates were allowed to stand at room temperature for 1 hr. Color development was carried out using SIGMA104 (Sigma-Aldrich) diluted to 1 mg/mL with a substrate buffer (50 mM $NaHCO_3$, pH9.8) containing 10 mM $MgCl_2$, and absorbance was measured at 405 nm using Benchmark Plus (Bio-Rad).

30 The binding activities of an antibody to shMpl-FLAG and hMPL-Fc were determined by ELISA. ELISA plates were coated with 1 μg/mL of purified shMpl-FLAG or hMPL-Fc, and blocked with a diluent buffer. Hybridoma culture supernatants were added to the plates, and the plates were allowed to stand at room temperature for 1 hr. Then, 35 alkaline phosphatase-labeled anti-mouse IgG antibodies (Zymed) were added to the plates. Color development was similarly carried out

using the above method. Following a one-hour coloring reaction at room temperature, absorbance was measured at 405 nm and EC_{50} values were computed using GraphPad Prism.

5   CHO-human Mpl cells and CHO-monkey Mpl cells were harvested, and suspended in FACS Buffer (1% FBS/ PBS) to a final concentration of $1x\, 10^6$ cells/mL. The suspensions were aliquoted into Multiscreen (Millipore) at 100 µl/well, and the culture supernatants were removed by centrifugation. Culture supernatants diluted to 5 µg/mL were added to the plates and incubated on ice for 30 min. The cells were

10   washed once with FACS buffer, and incubated on ice for 30 min following the addition of an FITC-labeled anti-mouse IgG antibody (Beckman Coulter). After incubation, the mixture was centrifuged at 500 rpm for 1 min. The supernatants were removed, and then the cells were suspended in 400 µL of FACS buffer. The samples were analyzed by flow

15   cytometry using EPICS ELITE ESP (Beckman Coulter). An analysis gate was set on the forward and side scatters of a histogram to include viable cell populations.

     Agonistic activities of an antibody were evaluated using BaF3-human Mpl and BaF3-monkey Mpl which proliferate in a

20   TPO-dependent manner. Cells of each cell line were suspended at $4x\, 10^5$ cells/ml in RPMI 1640/10% FBS (Invitrogen), and each suspension was aliquoted into a 96-well plate at 60µl/well. A 40-µL aliquot of rhTPO (R&D) and hybridoma culture supernatants prepared at various concentrations was added into each well. The plates were then

25   incubated at 37°C under 5% CO_2 for 24 hr. A 10-µL aliquot of the Cell Count Reagent SF (Nacalai Tesque) was added into each well. After incubation for 2 hr, absorbance was measured at 450 nm (and at 655 nm as a control) using a Benchmark Plus. EC_{50} values were calculated using GraphPad Prism.

30   The above analysis yielded a total of 163 clones of mouse monoclonal antibodies that bind to human Mpl.

     Among the anti-human Mpl antibodies to be described, TA136 was established from mice immunized with BaF3-human Mpl and the others were established from mice immunized with shMpl-Flag.

35

1.7 Purification of anti-human Mpl antibodies

Anti-human Mpl antibodies were purified from hybridoma culture supernatants as described below.

After the culture supernatants were loaded onto HiTrap protein G HP columns (Amersham Biosciences) for adsorption, the antibodies were eluted with 0.1 M glycine-HCl (pH2.7) Buffer. Immediately after elution, the fractions were neutralized with 1 M Tris-HCl Buffer (pH9.0), and dialyzed against PBS to replace the buffer for one day.

1.8 Determination of epitopes for the anti-human Mpl antibody VB22B

Since the anti-human Mpl antibody VB22B can be used for Western blotting, a GST-fusion protein containing a partial sequence of human Mpl was constructed for VB22B epitope analysis. MG1 (Gln26 to Trp491) and MG2 (Gln26 to Leu274) regions were each amplified by PCR, and cloned into pGEX-4T-3 (Amersham Biosciences) to be expressed as GST fusion proteins. The resulting plasmid DNAs were transformed into DH5α to give transformants. A final concentration of 1 mM IPTG was added to the transformants in their logarithmic growth phase to induce the expression of GST fusion proteins. The bacterial cells were harvested after two hours of incubation. The cells were lysed by sonication. The lysates were centrifuged in XL-80 Ultracentrifuge (Beckman, Rotor 70.1Ti) at 35,000 rpm for 30 min. The culture supernatants were removed, and then the fusion proteins were purified using GST Purification Modules (Amersham Biosciences). The samples were separated by 10%-SDS-PAGE, and then transferred onto a PVDF membrane. The membrane was analyzed by the murine antibody VB22B in Western Btotting. VB22B was found to recognize both MG-1 and MG-2, indicating that the VB22B epitope is located in the (Gln26 to Leu274) region.

Then, GST fusion proteins containing the respective regions of human Mpl: MG3 (Gln26 to Ala189), MG4 (Gln26 to Pro106), MG5 (Gln26 to Glu259), and MG6 (Gln26 to Gly245) were prepared and analyzed by Western blotting using the same procedure described above. VB22B was found to recognize MG5 and MG6, but not MG3 and MG4. This suggests that the VB22B epitope is located within the (Ala189 to Gly245) region. In addition, GST was fused with MG7 (Gln26 to Ala231) and MG8 (Gln26 to Pro217) to prepare GST fusion proteins. VB22B recognized MG7 but

not MG8, suggesting that the VB22B epitope is located in the (Gln217 to Ala231) region. Furthermore, GST fusion protein containing MG10 (Gln213 to Ala231) was recognized by VB22B, suggesting that the VB22B epitope is located within the limited region of 19 amino acids between Gln213 and Ala231.

5

1.9 Kinetic analyses of the antigen-antibody reaction for anti-human Mpl antibody VB22B

Since the anti-human Mpl antibody VB22B binds to soluble recombinant Mpl, kinetic analyses of the antigen-antibody reaction between VB22B IgG and human Mpl-IgG Fc fusion protein were carried out as described in Example 1.4. The Sensor Chip CM5 (Biacore) was placed in Biacore 2000 (Biacore), and human Mpl-IgG Fc fusion protein was immobilized onto the chip by amine-coupling methods. Then, 1.25 to 20 µg/mL of VB22B IgG solution was prepared using HBS-EP Buffer (Biacore), and injected over the chip surface for 2 min to reveal the binding region. Then, HBS-EP Buffer was injected over the chip surface for 2 min to reveal the dissociation region. VB22B IgG bound to the human Mpl-IgG Fc fusion protein on the sensor chip was removed by injecting 10 mM NaOH over the sensor chip for 15 sec, and the chip was recovered. HBS-EP Buffer was used as the running buffer, and the flow rate was 20 µL/min. Using the BIAevaluation Version 3.1 (Biacore) software, the reaction rate constant at each concentration was calculated from the sensorgrams. The dissociation constant (KD) for VB22B IgG was determined to be $1.67 \pm 0.713 \times 10^{-9}$ M.

[Example 2] Preparation of single-chain anti-human Mpl antibodies

Among the prepared anti-human Mpl antibodies, 23 types of antibodies, which exhibit higher binding activities and agonistic activities, were selected to construct expression systems for single-chain antibodies using genetic engineering techniques. An exemplary method for constructing a single-chain antibody derived from the anti-human Mpl antibody VB22B is described below.

2.1 Cloning of the anti-human Mpl antibody variable region

The variable region was amplified by RT-PCR using total RNA

extracted from hybridomas producing anti-human Mpl antibodies. Total RNA was extracted from 1x 10^7 hybridoma cells using the RNeasy Plant Mini Kit (QIAGEN).

5   A 5'-terminal fragment of the gene was amplified from 1 µg of total RNA by the SMART RACE cDNA Amplification Kit (Clontech), using a synthetic oligonucleotide MHC-IgG2b (SEQ ID NO: 166) complementary to mouse IgG2b constant region or a synthetic oligonucleotide kappa (SEQ ID NO: 167) complementary to mouse κ chain constant region. Reverse transcription was carried out at 42°C for 1.5 hr.

10   The composition of the PCR reaction solution (50 µL in total) is shown below.

10x Advantage 2 PCR Buffer (Clontech)	5 µL
10x Universal Primer A Mix (Clontech)	5 µL
dNTPs (dATP, dGTP, dCTP, and dTTP) (Clontech)	0.2 mM
Advantage 2 Polymerase Mix (Clontech)	1 µL
Reverse transcription product	2.5 µL
Synthetic oligonucleotide, MHC-IgG2b or kappa	10 pmol

The PCR reaction conditions were:
94°C (initial temperature) for 30 sec;
15   five cycles of 94°C for 5 sec and 72°C for 3 min;
five cycles of 94°C for 5 sec, 70°C for 10 sec, and 72°C for 3 min;
25 cycles of 94°C for 5 sec, 68°C for 10 sec, and 72°C for 3 min;
and final extension was at 72°C for 7 min.

The PCR products were purified from agarose gel using the
20   QIAquick Gel Extraction Kit (QIAGEN), and cloned into a pGEM-T Easy Vector (Promega). The nucleotide sequence was then determined using the ABI 3700 DNA Analyzer (Perkin Elmer).

The nucleotide sequence of cloned VB22B H chain variable region (hereinafter abbreviated as "VB22B-VH") is shown in SEQ ID NO: 117,
25   and its amino acid sequence is shown in SEQ ID NO: 118. The nucleotide sequence of the L chain variable region (hereinafter abbreviated as "VB22B-VL") is shown in SEQ ID NO: 119, and its amino acid sequence is shown in SEQ ID NO: 120.

2.2 Preparation of expression vectors for anti-human Mpl diabodies

The gene encoding VB22B single-chain Fv (hereinafter abbreviated as "VB22B diabody") containing a five-amino acid linker sequence was constructed, by linking a nucleotide sequence encoding a $(Gly_4Ser)_1$ linker to the VB22B-VH-encoding gene at its 3' end and to the VB22B-VL-encoding gene at its 5' end; both of which have been amplified by PCR.

The VB22B-VH forward primer, 70·115HF, (SEQ ID NO: 168) was designed to contain an EcoRI site. The VB22B-VH reverse primer, 33·115HR, (SEQ ID NO: 169) was designed to hybridize to a DNA encoding the C terminus of VB22B-VH, and to have a nucleotide sequence encoding the $(Gly_4Ser)_1$ linker and a nucleotide sequence hybridizing to the DNA encoding the N terminus of VB22B-VL. The VB22B-VL forward primer, 33·115LF, (SEQ ID NO: 170) was designed to have a nucleotide sequence encoding the N terminus of VB22B-VL, a nucleotide sequence encoding the $(Gly_4Ser)_1$ linker, and a nucleotide sequence encoding the C terminus of VB22B-VH. The VB22B-VL reverse primer, 33·115LR, (SEQ ID NO: 171) was designed to hybridize to a DNA encoding the C terminus of VB22B-VL and to have a nucleotide sequence encoding a FLAG tag (Asp Tyr Lys Asp Asp Asp Asp Lys/SEQ ID NO: 172) and a NotI site.

In the first round of PCR, two PCR products: one containing VB22B-VH and a linker sequence, and the other containing VB22B-VL and the identical linker sequence, were synthesized by the procedure described below.

The composition of the PCR reaction solution (50 µL in total) is shown below.

10x PCR Buffer (TaKaRa)	5 µL
dNTPs (dATP, dGTP, dCTP, and dTTP) (TaKaRa)	0.4 mM
DNA polymerase TaKaRa Ex Taq (TaKaRa)	2.5 units
pGEM-T Easy vector comprising VB22B-VH or VB22B-VL gene	10 ng
Synthetic oligonucleotides, 70·115HF and 33·115HR, or 33·115LF and 33·115LR	10 pmol

The PCR reaction conditions were:

94°C (initial temperature) for 30 sec;

five cycles of: 94°C for 15 sec and 72°C for 2 min;

five cycles of 94°C for 15 sec and 70°C for 2 min;

28 cycles of 94°C for 15 sec and 68°C for 2 min;

5　and final extension was at 72°C for 5 min.

After the PCR products of about 400 bp were purified from agarose gel using the QIAquick Gel Extraction Kit (QIAGEN), the second-round PCR was carried out using aliquots of the respective PCR products according to the protocol described below.

10　The composition of the PCR reaction solution (50 μL in total) is shown below.

10x PCR Buffer (TaKaRa)	5 μL
dNTPs (dATP, dGTP, dCTP, and dTTP) (TaKaRa)	0.4 mM
DNA polymerase TaKaRa Ex Taq (TaKaRa)	2.5 unit
First-round PCR products (two types)	1 μL
Synthetic oligonucleotides, 70·115HF and 33·115LR	10 pmol

The reaction conditions were:

94°C (initial temperature) for 30 sec;

15　five cycles of 94°C for 15 sec and 72°C for 2 min;

five cycles of 94°C for 15 sec and 70°C for 2 min;

28 cycles of 94°C for 15 sec and 68°C for 2 min;

and final extension was at 72°C for 5 min.

The PCR products of about 800 bp were purified from agarose gel

20　using the QIAquick Gel Extraction Kit (QIAGEN), and then digested with EcoRI and NotI (both from TaKaRa). The resulting DNA fragments were purified using the QIAquick PCR Purification Kit (QIAGEN), and then cloned into pCXND3 to prepare pCXND3-VB22B db.

25　2.3 Preparation of expression vectors for anti-human Mpl antibody sc(Fv)2

To prepare expression plasmids for the modified antibody [sc(Fv)2] comprising two units of H chain variable region and two units of L chain variable region derived from VB22B, the

30　above-described pCXND3-VB22B db was modified by PCR using the

procedure shown below. The process for constructing the sc(Fv)2 gene is illustrated in Fig. 1.

First, PCR method was carried out to amplify (a) the VB22B-VH-encoding gene in which a nucleotide sequence encoding a 15-amino acid linker (Gly$_4$Ser)$_3$ was added to its 3' end; and (b) the VB22B-VL-encoding gene containing the identical linker nucleotide sequence added to its 5' end. The desired construct was prepared by linking these amplified genes. Three new primers were designed in this construction process. The VB22B-VH forward primer, VB22B-fpvu, (primer A; SEQ ID NO: 173) was designed to have an EcoRI site at its 5' end and to convert Gln22 and Leu23 of VB22B db into a PvuII site. The VB22B-VH reverse primer, sc-rL15, (primer B; SEQ ID NO: 174) was designed to hybridize to a DNA encoding the C terminus of VB22B-VH, and to have a nucleotide sequence encoding the (Gly$_4$Ser)$_3$ linker, as well as a nucleotide sequence hybridizing to a DNA encoding the N terminus of VB22B-VL. The VB22B-VL forward primer, sc-fL15, (primer C; SEQ ID NO: 175) was designed to have a nucleotide sequence encoding the N terminus of VB22B-VL, a nucleotide sequence encoding the (Gly$_4$Ser)$_3$ linker, and a nucleotide sequence encoding the C terminus of VB22B-VH.

In the first-round PCR, two PCR products: one comprising VB22B-VH and a linker sequence, and the other comprising VB22B-VL and the identical linker sequence, were synthesized by the procedure described below.

The composition of the PCR reaction solution (50 µL in total) is shown below.

10x PCR Buffer (TaKaRa)	5 µL
dNTPs (dATP, dGTP, dCTP, and dTTP) (TaKaRa)	0.4 mM
DNA polymerase TaKaRa Ex Taq (TaKaRa)	2.5 units
pCXND3-VB22B db	10 ng
Synthetic oligonucleotides, VB22B-fpvu, sc-rL15 or sc-fL15, and 33·115LR (primer D)	10 pmol

The reaction conditions were:
94°C (initial temperature) for 30 sec;

five cycles of 94°C for 15 sec and 72°C for 2 min;
five cycles of 94°C for 15 sec and 70°C for 2 min;
28 cycles of 94°C for 15 sec and 68°C for 2 min;
and final extension was at 72°C for 5 min.

5   After the PCR products of about 400 bp were purified from agarose gel using the QIAquick Gel Extraction Kit (QIAGEN), the second-round PCR was carried out using aliquots of the respective PCR products according to the protocol described below.

       The composition of the PCR reaction solution (50 µL in total)

10   is shown below.

10x PCR Buffer (TaKaRa)	5 µL
dNTPs (dATP, dGTP, dCTP, and dTTP) (TaKaRa)	0.4 mM
DNA polymerase TaKaRa Ex Taq (TaKaRa)	2.5 units
First-round PCR product (two types)	1 µL
Synthetic oligonucleotide, 70·115HF and 33·115LR	10 pmol

       The reaction conditions were:
94°C (initial temperature) for 30 sec;
five cycles of 94°C for 15 sec and 72°C for 2 min;

15   five cycles of 94°C for 15 sec and 70°C for 2 min;
28 cycles of 94°C for 15 sec and 68°C for 2 min;
and final extension was at 72°C for 5 min.

       The PCR products of about 800 bp were purified from agarose gel using the QIAquick Gel Extraction Kit (QIAGEN), and then digested

20   with EcoRI and NotI (both from TaKaRa). The resulting DNA fragments were purified using the QIAquick PCR Purification Kit (QIAGEN), and then cloned into pBacPAK9 (Clontech) to construct pBacPAK9-scVB22B.

       A fragment to be inserted into the PvuII site of pBacPAK9-scVB22B was prepared. Specifically, the fragment has a

25   PvuII recognition site at both ends and a nucleotide sequence, in which a gene encoding the VB22B-VH N-terminus is linked, via a $(Gly_4Ser)_3$ linker-encoding nucleotide sequence, to a gene encoding the amino acid sequence of an N terminus-deleted VB22B-VH linked to VB22B-VL via the $(Gly_4Ser)_3$ linker. Two primers were newly designed

30   to prepare the fragment by PCR. The forward primer for the fragment

of interest, Fv2-f (primer E; SEQ ID NO: 176), was designed to have a PvuII site at its 5' end and a VB22B-VH 5'-end sequence. The reverse primer for the fragment of interest, Fv2-r (primer F; SEQ ID NO: 177), was designed to hybridize to a DNA encoding the C terminus of VB22B-VL, and to have a PvuII site, a nucleotide sequence encoding the $(Gly_4Ser)_3$ linker, and a nucleotide sequence hybridizing to a DNA encoding the N terminus of VB22B-VH. PCR was carried out using pBacPAK9-scVB22B as a template as described below.

The composition of the PCR reaction solution (50 μL in total) is shown below.

10x PCR Buffer (TaKaRa)	5 μL
dNTPs (dATP, dGTP, dCTP, and dTTP) (TaKaRa)	0.4 mM
DNA polymerase TaKaRa Ex Taq (TaKaRa)	2.5 units
pBacPAK9-scVB22B	10 μg
Synthetic oligonucleotide, Fv2-f and Fv2-r	10 pmol

The reaction conditions were:
94°C (initial temperature) for 30 sec;
five cycles of 94°C for 15 sec and 72°C for 2 min;
five cycles of 94°C for 15 sec and 70°C for 2 min;
28 cycles of 94°C for 15 sec and 68°C for 2 min;
and final extension was at 72°C for 5 min.

The PCR products of about 800 bp were purified from agarose gel using the QIAquick Gel Extraction Kit (QIAGEN), and then cloned into the pGEM-T Easy Vector (Promega). After sequencing, the plasmid was digested with PvuII (TaKaRa), and the fragment of interest was recovered. The recovered fragment was ligated to pBacPAK9-scVB22B pre-digested with PvuII (TaKaRa) to construct pBacPAK9-VB22B sc(Fv)2. After the resulting vector was digested with EcoRI and NotI (both from TaKaRa), the fragment of about 1,600 bp was purified from agarose gel using the QIAquick Gel Extraction Kit (QIAGEN). The fragment was then cloned into a pCXND3 expression vector to construct pCXND3-VB22B sc(Fv)2.

2.4 Expression of single-chain anti-human Mpl antibody in animal cells

A cell line stably expressing the single-chain antibody was prepared from CHO-DG44 cells as described below. Gene transfer was achieved by electroporation using a Gene Pulser II (Bio-Rad). An expression vector (25 µg) and 0.75 mL of CHO-DG44 cells suspended

5   in PBS (1x 10^7 cells/mL) were mixed. The resulting mixture was cooled on ice for 10 min, transferred into a cuvette, and pulsed at 1.5-kV and 25 µFD. After a ten-minute restoration period at room temperature, the electroporated cells were plated in CHO-S-SFMII medium (Invitrogen) containing 500 µg/mL Geneticin (Invitrogen). CHO cell

10 lines expressing the single-chain antibody were established through selection. A cell line stably expressing VB22B sc(Fv)2 and its culture supernatants were obtained by this method.

The transient expression of the single-chain antibody was achieved using COS7 cells as described below. An expression vector

15 (10 µg) and 0.75 mL of COS7 cells suspended in PBS (1x 10^7 cells/mL) were mixed. The resulting mixture was cooled on ice for 10 min, transferred into a cuvette, and then pulsed at 1.5-kV and 25 µFD. After a ten-minute restoration period at room temperature, the electroporated cells were plated in DMEM/10% FBS medium (Invitrogen).

20 The cells were incubated overnight and then washed with PBS. CHO-S-SFMII medium was added and the cells were cultured for about three days. The culture supernatants for preparing the VB22B diabody were thus prepared.

25 2.5 Quantitation of single-chain anti-human Mpl antibodies in culture supernatants

The culture supernatant concentration of the single-chain anti-human Mpl antibody transiently expressed in COS cells was determined using surface plasmon resonance. A sensor chip CM5

30 (Biacore) was placed in Biacore 2000 (Biacore). ANTI-FLAG® M2 Monoclonal Antibody (Sigma-Aldrich) was immobilized onto the chip. An appropriate concentration of sample was injected over the chip surface at a flow rate of 5 mL/sec, and 50 mM diethylamine was used to dissociate the bound antibody. Changes in the mass during sample

35 injection were recorded, and the sample concentration was calculated from the calibration curve prepared using the mass changes of a

standard sample. db12E10 (see WO 02/33073 and WO 02/33072) was used as the diabody standard, and 12E10 sc(Fv)2 which has the same gene structure as that of sc(Fv)2 was used as the sc(Fv)2 standard.

5   2.6 Purification of anti-human Mpl diabodies and single-chain antibodies

The culture supernatants of VB22B diabody-expressing COS7 cells or CHO cells was loaded onto an Anti-Flag M2 Affinity Gel (Sigma-Aldrich) column equilibrated with a 50 mM Tris-HCl buffer
10   (pH7.4) containing 150 mM NaCl and 0.05% Tween 20. The absorbed antibodies were eluted with 100 mM glycine-HCl (pH3.5). The fractions eluted were immediately neutralized with 1 M Tris-HCl (pH8.0), and loaded onto a HiLoad 26/60 Superdex 200 pg (Amersham Biosciences) column for gel filtration chromatography. PBS/0.01%
15   Tween 20 was used in the gel filtration chromatography.

VB22B sc(Fv)2 was purified from the culture supernatants of VB22B sc(Fv)2-expressing COS7 cells or CHO cells under the same conditions used for purifying the diabodies. A large-scale preparation of VB22B sc(Fv)2 was prepared by loading the CHO cell
20   culture supernatants onto a Macro-Prep Ceramic Hydroxyapatite Type I (Bio-Rad) column equilibrated with a 20 mM phosphate buffer (pH6.8), and eluting the VB22B sc(Fv)2 in a stepwise manner with 250 mM phosphate buffer (pH6.8). The eluted fraction was concentrated on an ultrafilter, and then fractionated by gel filtration
25   chromatography using a HiLoad 26/60 Superdex 200 pg (Amersham Biosciences) column, and a fraction corresponding to the molecular weight range of about 40 kD to 70 kD was obtained. The fraction was loaded onto an Anti-Flag M2 Affinity Gel column equilibrated with a 50 mM Tris-HCl buffer (pH7.4) containing 150 mM NaCl and 0.05% Tween
30   20. The absorbed antibody was eluted with 100 mM glycine-HCl (pH3.5). The eluted fraction was immediately neutralized with 1 M Tris-HCl (pH8.0), and loaded onto a HiLoad 26/60 Superdex 200 pg (Amersham Biosciences) column for gel filtration chromatography. 20 mM acetate buffer (pH6.0) containing 150 mM NaCl and 0.01% Tween 80 was used
35   in the gel filtration chromatography. In each purification step, the presence of the diabody and sc(Fv)2 in the samples was confirmed by

SDS-PAGE and Western blotting using an anti-Flag antibody (Sigma-Aldrich). Specifically, obtained fractions corresponding to each peak were subjected to the electrophoresis according to the method described by Laemli, and then stained using Coomassie Brilliant Blue. As a result, single band was detected apparently at about 29 kDa for the diabody; while single band was detected apparently at about 55 kDa for sc(Fv)2.

2.7 Binding activity analyses of single-chain anti-human Mpl antibodies by flow cytometry

CHO-human Mpl, CHO-monkey Mpl, and CHO-mouse Mpl cells were recovered and suspended in FACS buffer (1% FBS/PBS) to a final concentration of 1x 10^6 cells/mL. Cell suspensions were aliquoted at 100-μL/well into the Multiscreen-HV Filter Plates (Millipore). After centrifugation, the supernatant was removed. An appropriate concentration of diabody or sc(Fv)2 was added into each well and incubated on ice for 30 min. The cells were washed once with 200 μL of FACS buffer, and incubated on ice for 30 min following the addition of 10 μg/mL ANTI-FLAG® M2 Monoclonal Antibody (Sigma-Aldrich). The cells were then washed once with 200 μL of FACS buffer, and a 100x-diluted FITC-labeled anti-mouse IgG antibody (Beckman Coulter) was added to the plate. The plate was incubated on ice for 30 min. After centrifugation, the supernatant was removed. The cells were suspended in 400 μL of FACS Buffer, and then analyzed by flow cytometry using EPICS ELITE ESP (Beckman Coulter). An analysis gate was set on the forward and side scatters of a histogram to include viable cell populations.

The binding activity of the purified VB22B sc(Fv)2 to various Mpl molecules expressed in CHO cells was determined (Fig. 2). VB22B sc(Fv)2 was found to specifically bind to CHO-human Mpl and CHO-monkey Mpl but not to the host cell CHO or CHO-mouse Mpl. This binding characteristic of VB22B sc(Fv)2 is comparable to those of VB22B IgG, indicating that the antibody binding site remains unaltered by converting whole IgG to minibody.

2.8 Analyses of TPO-like agonistic activity for single-chain

anti-human Mpl antibodies

TPO-like agonistic activity was assessed using BaF3-human Mpl or BaF3-monkey Mpl that proliferate in a TPO-dependent manner.

Cells from each cell line were washed twice with RPMI 1640/1% FBS (fetal bovine serum) (Invitrogen), and then suspended in RPMI 1640/10% FBS to a concentration of $4x\ 10^5$ cells/mL. Cell suspensions were aliquoted at 60-μL/well into a 96-well plate. Various concentrations of rhTPO (R&D) and COS7 culture supernatants or purified samples were prepared, and a 40-μL aliquot was added into each well. The plates were then incubated at 37°C under 5% CO_2 for 24 hr. Immediately after a 10-μL aliquot of WST-8 reagent (Cell Count Reagent SF; Nacalai Tesque) was added into each well, absorbance was measured at 450 nm (and at 655 nm as a control) using Benchmark Plus. After two hours of incubation, absorbance was again measured at 450 nm (and at 655 nm as a control). The WST-8 reagent changes colors at 450 nm in a color reaction that reflects the viable cell count. The TPO-like agonistic activity was assessed using the change in absorbance during the two-hour incubation as an index. EC_{50} values were computed using GraphPad Prism.

TPO-like agonistic activity was assayed using the human leukemia cell line M-07e (purchased from DSMZ) which proliferates TPO-dependently. M-07e cells were washed twice with RPMI 1640/1% FBS, and then suspended in RPMI 1640/10% FBS to a concentration of $5x\ 10^5$ cells/mL. The resulting cell suspension was aliquoted at 50-μL/well into a 96-well plate. Various concentrations of rhTPO and COS7 culture supernatants or purified samples were prepared, and a 50-μL aliquot was added into each well. The plates were then incubated at 37°C under 5% CO_2 for 48 hr. Immediately after a 10-μL aliquot of WST-8 reagent (Cell Count Reagent SF; Nacalai Tesque) was added to each well, absorbance of was measured at 450 nm (and at 655 nm as a control) using a Benchmark Plus. After four hours of incubation, absorbance was again measured at 450 nm (and at 655 nm as a control). The TPO-like agonistic activity was assayed using the change in absorbance during the four-hour incubation as an index.

Purified VB22B IgG, VB22B diabody, and VB22B sc(Fv)2 were assayed for their TPO-like agonistic activities using BaF3-human Mpl,

BaF3-monkey Mpl, and M-07e. The results are shown in Figs. 3, 4, and
5, respectively. The presence of bivalent antigen-binding domains
in a single antibody molecule is essential for its agonistic activity.
The distance and angle between two antigen-binding domains can also
be important factors (see WO 02/33073 and WO 02/33072). Similar
results were obtained for the newly isolated anti-human Mpl antibodies.
Specifically, the agonistic activities of VB22B diabody and VB22B
sc(Fv)2 (EC_{50} = 61 pM and 27 pM in BaF3-human Mpl, respectively) were
higher than that of VB22B IgG (EC_{50} > 30 nM in BaF3-human Mpl), and
were equivalent to or higher than that of the naturally-occurring
human TPO ligand (EC_{50} = 76 pM in BaF3-human Mpl). The VB22B diabody
activity was lower than that of VB22B sc(Fv)2. This suggests that
the structure of a single-chain antibody is greatly altered by its
molecular shape and the length of the linker sequence, which in turn
changes the agonistic activity. Sixteen types of the single-chain
anti-human Mpl antibodies were obtained, each exhibiting a high
agonistic activity. The amino acid sequences of the H chain and L
chain variable regions of the representative antibodies are shown
in Figs. 6 and 7, respectively.

2.9 Humanization of single-chain anti-human Mpl antibody

Antibody sequence data for the humanization of VB22B sc(Fv)2
were obtained from the Kabat Database
(ftp://ftp.ebi.ac.uk/pub/databases/kabat/), and homology searches
were carried out independently for the H chain variable region and
the L chain variable region. As a result, the H chain variable region
was found to be highly homologous to DN13 (Smithson S. L. et *al.*,
Mol Immunol. 36, 113-124 (1999)). The L chain variable region was
found to be highly homologous to ToP027 (Hougs L. et *al.*, J. Immunol.
162, 224-237 (1999)). Humanized antibodies were prepared by
inserting a complementarity-determining region (hereinafter
abbreviated as "CDR") into the framework regions (hereinafter
abbreviated as "FR") of the above antibodies. The humanized antibody
sc(Fv)2 was expressed in CHO-DG44 cells, and its agonistic activity
was assessed using BaF3-human Mpl. The agonistic activity was used
as an index to generate a humanized VB22B sc(Fv)2 which has agonistic

activity equivalent to that of murine VB22B sc(Fv)2 by replacing one or more amino acids in its framework region.

Specifically, synthetic oligo-DNAs of approximately 50 nucleotides in length were designed as to make 20 of these nucleotides available for hybridization, and the synthetic oligo-DNAs were assembled by PCR to prepare genes that encode the respective variable regions. Using the resulting genes, sc(Fv)2 was similarly prepared by the method described in Example 2.3. The respective DNAs were cloned into a pCXND3expression vector to construct expression vectors pCXND3-hVB22B p-z sc(Fv)2, pCXND3-hVB22B g-e sc(Fv)2, pCXND3-hVB22B e sc(Fv)2, pCXND3-hVB22B u2-wz4 sc(Fv)2, and pCXND3-hVB22B q-wz5 sc(Fv)2, to which the humanized VB22B sc(Fv)2 is inserted. The nucleotide sequences and the amino acid sequences of the fragments in each plasmid are shown below.

Plasmid name	Nucleotide sequence	Amino acid sequence
hVB22B p-z sc(Fv)2	SEQ ID NO: 1	SEQ ID NO: 2
hVB22B g-e sc(Fv)2	SEQ ID NO: 253	SEQ ID NO: 254
hVB22B e sc(Fv)2	SEQ ID NO: 259	SEQ ID NO: 260
hVB22B u2-wz4 sc(Fv)2	SEQ ID NO: 286	SEQ ID NO: 287
hVB22B q-wz5 sc(Fv)2	SEQ ID NO: 292	SEQ ID NO: 293
Murine VB22B sc(Fv)2	SEQ ID NO: 263	SEQ ID NO: 264

The plasmids were expressed in CHO-DG44 cells and the culture supernatants were recovered by the method described in Example 2.4. Since the humanized VB22B sc(Fv)2 does not contain a Flag tag, its purification from the culture supernatant was performed using a MG10-GST fusion protein. MG10 (Gln213 to Ala231) is one of the epitopes recognized by VB22B, as described in Example 1.8. The MG10-GST fusion protein was purified using Glutathione Sepharose 4B (Amersham Biosciences) according to the supplier's protocol. Then, the purified MG10-GST fusion protein was immobilized onto a HiTrap NHS-activated HP Column (Amersham Biosciences) to prepare an affinity column, according to the supplier's protocol. The culture supernatant of CHO cells expressing the humanized VB22B sc(Fv)2 was

loaded onto the MG10-GST fusion protein-immobilized column, which has been equilibrated with 50 mM Tris-HCl (pH7.4)/150 mM NaCl/0.01% Tween 80. The adsorbed humanized VB22B sc(Fv)2 was eluted with 100 mM glycine-HCl (pH3.5)/0.01% Tween 80. Immediately after elution,

5   the eluted fraction was neutralized with 1 M Tris-HCl (pH7.4), and was further subjected to gel filtration chromatography using a HiLoad 16/60 Superdex 200 pg (Amersham Biosciences). 20 mM citrate buffer (pH7.5) containing 300 mM NaCl and 0.01% Tween 80 was used in the gel filtration chromatography. The TPO-like agonistic activities of

10   the purified samples were similarly determined using the method described in Example 2.8. The TPO-like agonistic activities of the purified murine VB22B sc(Fv)2, hVB22B p-z sc(Fv)2, hVB22B u2-wz4 sc(Fv)2, hVB22B q-wz5 sc(Fv)2, and humanized hVB22B e sc(Fv)2 and hVB22B g-e sc(Fv)2 were assessed in BaF3-human Mpl. The results are

15   shown in Figs. 19, 20, and 21. The humanized VB22B sc(Fv)2 showed comparable agonistic activities, suggesting that the humanization has no influence on the activity.

2.10 Kinetic analyses of the antigen-antibody reaction for anti-human

20   Mpl antibodies: VB22B IgG, VB22B sc(Fv)2, and humanized VB22B sc(Fv)2
     Using the soluble recombinant Mpl-binding characteristic of anti-human Mpl antibody VB22B, kinetic analyses of the antigen-antibody reactions between the MG10 (Gln 213 to Ala 231)-GST fusion protein and each of VB22B IgG, VB22B sc(Fv)2, and humanized

25   VB22B sc(Fv)2 were carried out as described in Example 1.8. The Sensor Chip CM5 (Biacore) was placed in Biacore 3000 (Biacore), and MG10-GST fusion protein was immobilized onto the chip by amine-coupling methods. HBS-EP Buffer (Biacore) was used as the running buffer, and the flow rate was 20 µL/min. 5.5 to 175.0 nM of VB22B IgG solution was prepared

30   using HBS-EP Buffer, and injected over each of the chip surfaces for 2 min to obtain the binding region at the respective concentrations. Then, dissociation region for the 2 minutes was measured. VB22B IgG bound to the MG10-GST fusion protein on the sensor chip was removed by injecting 20 mM HCl over the sensor chip for 1 min, and the chip

35   was recovered. Similarly, 4.7 to 150.1 nM of VB22B sc(Fv)2, 5.3 to 168.9 nM of hVB22B q-wz5 sc(Fv)2, and 4.9 to 156.8 nM of hVB22B u2-wz4

sc(Fv)2 were prepared and injected over the chip surfaces onto which MG10-GST fusion protein was immobilized, and the measurement was carried out.

All the antibodies used were bivalent antibodies, and thus the sensorgrams at each concentration were obtained in the presence of both monovalent and bivalent bindings. In this context, the reaction rate constant was determined as that for the monovalent antibody by analysis using the Bivalent analyte model of BIAevaluation ver.3.1 software (Biacore). The above analysis was carried out in triplicates for each antibody. The binding rate constant (ka), dissociation rate constant (kd), and dissociation constant (KD) were determined as those for the monovalent antibody by the procedure described above. The constants are indicated below in Table 1. The dissociation constants (KD) for VB22B IgG, VB22B sc(Fv)2, hVB22B q-wz5 sc(Fv)2, and hVB22B u2-wz4 sc(Fv)2 were determined to be 1.15×10^{-8} M, 1.17×10^{-8} M, 1.36×10^{-8} M, and 1.02×10^{-8} M, respectively, showing nearly equivalent binding activities towards the MG10-GST fusion protein.

Table 1   Kinetic analyses of the antigen-antibody reaction for anti-human Mpl antibodies

Antibody Name	ka (1/Ms) [x10^5]	kd (1/s) [x10^{-3}]	KD (M) [x10^{-8}]
VB22B IgG	0.96 ± 0.78	1.10 ± 0.01	1.15 ± 0.09
VB22B sc(Fv)2	4.23 ± 0.22	4.91 ± 0.72	1.17 ± 0.23
hVB22B q-wz5 sc(Fv)2	3.76 ± 0.38	5.10 ± 0.56	1.36 ± 0.06
hVB22B u2-wz4 sc(Fv)2	6.08 ± 0.30	6.17 ± 0.23	1.02 ± 0.08

[Example 3]   Preparation of anti-Mpl diabodies by the AGS method
Anti-Mpl diabodies having agonistic activity were prepared by an Autocrine Growth Selection (AGS) method (see, WO 03/91424).

3.1 Construction of a retrovirus library
Spleens were isolated from MRL/lpr mice immunized with shMPL-Flag by the method described in Example 1.5, and homogenized

in TRIZOL Reagent (Invitrogen) using a Dounce homogenizer. After chloroform addition, the homogenized sample was shaken vigorously, the aqueous phase was removed and total RNA was extracted by isopropanol precipitation. mRNA was purified using a PolyATract

5 System 1000 (Promega). Reverse transcription of 2.5 µg mRNA was carried out at 42°C for 50 min using the Superscript First strand synthesis system for RT-PCR (Invitrogen) and the included oligo-dT primers to prepare cDNA.

The composition of the PCR reaction solution (250 µL) is shown

10 below.

10x KOD Plus Buffer (Toyobo)	25 µL
2 mM dNTPs (dATP, dGTP, dCTP, and dTTP) (Toyobo)	25 µL
2.5 mM MgSO$_4$ (Toyobo)	10 µL
KOD Plus (Toyobo)	7.5 µL
Reverse transcription products	25 µL
Mixed primers complementary to H chain or L chain variable region	500 pmol

The reaction conditions were:

98°C (initial temperature) for 3 min;

32 cycles of 98°C for 20 sec, 58°C for 20 sec, and 72°C for 30 sec;

15 and final extension was at 72°C for 6 min.

The H chain primer mix contained HS1 to HS19 (SEQ ID NOs: 178 to 196) and HA1 to HA4 (SEQ ID NOs: 197 to 200), which were mixed at the indicated ratios next to the sequence names in Table 2. The L chain primer mix contained LS1 to LS17 (SEQ ID NOs: 201 to 217),

20 Lslambda (SEQ ID NO: 218), LA1 to LA5 (SEQ ID NOs: 219 to 222), and Lalambda (SEQ ID NO: 223). The respective PCR products were purified from agarose gel using the QIAquick Gel Extraction Kit (QIAGEN). The H chain and L chain variable regions were linked via the (Gly$_4$Ser)$_1$ linker sequence by PCR using sc-S (SEQ ID NO: 224) and sc-AS (SEQ

25 ID NO: 225) as described below.

The composition of the PCR reaction solution (100 µL in total) is shown below.

10x KOD Plus Buffer (Toyobo)	10 µL

2 mM dNTPs (dATP, dGTP, dCTP, and dTTP) (Toyobo)	10 µL
2.5 mM MgSO₄ (Toyobo)	4 µL
KOD Plus (Toyobo)	2 µL
Fragment of H chain variable region	4 µL
Fragment of L chain variable region	4 µL

The first-round PCR conditions were:

94°C (initial temperature) for 3 min; and

seven cycles of 94°C for 1 min and 63°C for 4 min.

5   Then, sc-S and sc-AS (25 pmol each) were added to the first-round products.

The second-round PCR conditions were:

30 cycles of 94°C for 30 sec, 55°C for 2 min, and 72°C for 2 min; and final extension was at 72°C for 6 min.

10   The resulting product with an SfiI restriction site at both ends was purified using the QIAquick PCR Purification Kit (QIAGEN), and incubated with the SfiI restriction enzyme (TaKaRa) overnight at 50°C. The PCR product purified from agarose gel using the QIAquick Gel Extraction Kit (QIAGEN) was inserted into the SfiI site of the viral

15   vector pMX/IL3ssGFPHis.

The resulting plasmid was constructed by inserting a GFP gene, which has an EcoRI site, mouse IL-3 signal sequence and SfiI site at its 5' end; and an SfiI site, His tag sequence, termination codon, and NotI site at its 3' end, between the EcoRI and NotI sites on the

20   pMX viral vector (Onishi, M. *et al.*, Mol. Cell. Biol. 18, 3871-3879). The plasmid was introduced into the ElectroMAX DH10B T1 phage resistant cells (Invitrogen) by electroporation (settings: 2.5 kV, 25 µF, and 100Ω) using a Gene Pulser II (Bio-Rad). The cells were plated onto an LB-Agar plate containing 100 µg/mL ampicillin. After

25   overnight incubation, 1x 10⁷ colonies were obtained. Colonies were recovered from the plate and plasmids were then extracted using the QIAGEN Plasmid Maxi Kit (QIAGEN).

Table 2

```
SEQ ID NO:178 (HS1(4)) GCCCAGCCGGCCATGGCGGAKGTRMAGCTTCAGGAGTC
SEQ ID NO:179 (HS2(4)) GCCCAGCCGGCCATGGCGGAGGTBCAGCTBCAGCAGTC
SEQ ID NO:180 (HS3(3)) GCCCAGCCGGCCATGGCGCAGGTGCAGCTGAAGSASTC
SEQ ID NO:181 (HS4(4)) GCCCAGCCGGCCATGGCGGAGGTCCARCTGCAACARTC
SEQ ID NO:182 (HS5(7)) GCCCAGCCGGCCATGGCGCAGGTYCAGCTBCAGCARTC
SEQ ID NO:183 (HS6(2)) GCCCAGCCGGCCATGGCGCAGGTYCARCTGCAGCAGTC
SEQ ID NO:184 (HS7(1)) GCCCAGCCGGCCATGGCGCAGGTCCACGTGAAGCAGTC
SEQ ID NO:185 (HS8(2)) GCCCAGCCGGCCATGGCGGAGGTGAASSTGGTGGAATC
SEQ ID NO:186 (HS9(5)) GCCCAGCCGGCCATGGCGGAVGTGAWGYTGGTGGAGTC
SEQ ID NO:187 (HS10(2)) GCCCAGCCGGCCATGGCGGAGGTGCAGSKGGTGGAGTC
SEQ ID NO:188 (HS11(2)) GCCCAGCCGGCCATGGCGGAKGTGCAMCTGGTGGAGTC
SEQ ID NO:189 (HS12(2)) GCCCAGCCGGCCATGGCGGAGGTGAAGCTGATGGARTC
SEQ ID NO:190 (HS13(1)) GCCCAGCCGGCCATGGCGGAGGTGCARCTTGTTGAGTC
SEQ ID NO:191 (HS14(2)) GCCCAGCCGGCCATGGCGGARGTRAAGCTTCTCGAGTC
SEQ ID NO:192 (HS15(2)) GCCCAGCCGGCCATGGCGGAAGTGAARSTTGAGGAGTC
SEQ ID NO:193 (HS16(5)) GCCCAGCCGGCCATGGCGCAGGTTACTCTRAAAGWGTSTG
SEQ ID NO:194 (HS17(3.5)) GCCCAGCCGGCCATGGCGCAGGTCCAACTVCAGCARCC
SEQ ID NO:195 (HS18(0.7)) GCCCAGCCGGCCATGGCGGATGTGAACTTGGAAGTGTC
SEQ ID NO:196 (HS19(0.7)) GCCCAGCCGGCCATGGCGGAGGTGAAGGTCATCGAGTC
SEQ ID NO:197 (HA1(1)) GGAGCCGCCGCCGCCCGAGGAAACGGTGACCGTGGT
SEQ ID NO:198 (HA2(1)) GGAGCCGCCGCCGCCCGAGGAGACTGTGAGAGTGGT
SEQ ID NO:199 (HA3(1)) GGAGCCGCCGCCGCCCGCAGAGACAGTGACCAGAGT
SEQ ID NO:200 (HA4(1)) GGAGCCGCCGCCGCCCGAGGAGACGGTGACTGAGGT
SEQ ID NO:201 (LS1(1)) GGCGGCGGCGGCTCCGAYATCCAGCTGACTCAGCC
SEQ ID NO:202 (LS2(2)) GGCGGCGGCGGCTCCGAYATTGTTCTCWCCCAGTC
SEQ ID NO:203 (LS3(5)) GGCGGCGGCGGCTCCGAYATTGTGMTMACTCAGTC
SEQ ID NO:204 (LS4(3.5)) GGCGGCGGCGGCTCCGAYATTGTGYTRACACAGTC
SEQ ID NO:205 (LS5(4)) GGCGGCGGCGGCTCCGAYATTGTRATGACMCAGTC
SEQ ID NO:206 (LS6(7)) GGCGGCGGCGGCTCCGAYATTMAGATRAMCCAGTC
SEQ ID NO:207 (LS7(6)) GGCGGCGGCGGCTCCGAYATTCAGATGAYDCAGTC
SEQ ID NO:208 (LS8(1.5)) GGCGGCGGCGGCTCCGAYATYCAGATGACACAGAC
SEQ ID NO:209 (LS9(2)) GGCGGCGGCGGCTCCGAYATTGTTCTCAWCCAGTC
SEQ ID NO:210 (LS10(3.5)) GGCGGCGGCGGCTCCGAYATTGWGCTSACCCAATC
SEQ ID NO:211 (LS11(8)) GGCGGCGGCGGCTCCGAYATTSTRATGACCCARTC
SEQ ID NO:212 (LS12(8)) GGCGGCGGCGGCTCCGAYRTTKTGATGACCCARAC
SEQ ID NO:213 (LS13(6)) GGCGGCGGCGGCTCCGAYATTGTGATGACBCAGKC
SEQ ID NO:214 (LS14(2)) GGCGGCGGCGGCTCCGAYATTGTGATAACYCAGGA
SEQ ID NO:215 (LS15(2)) GGCGGCGGCGGCTCCGAYATTGTGATGACCCAGWT
SEQ ID NO:216 (LS16(1)) GGCGGCGGCGGCTCCGAYATTGTGATGACACAACC
SEQ ID NO:217 (LS17(1)) GGCGGCGGCGGCTCCGAYATTTTGCTGACTCAGTC
SEQ ID NO:218 (LSlambda(1)) GGCGGCGGCGGCTCCGATGCTGTTGTGACTCAGGAATC
SEQ ID NO:219 (LA1(4)) GGAATTCGGCCCCCGAGGCCTTGATTTCCAGCTTGG
SEQ ID NO:220 (LA2(4)) GGAATTCGGCCCCCGAGGCCTTTATTTCCAGCTTGG
SEQ ID NO:221 (LA4(4)) GGAATTCGGCCCCCGAGGCCTTTATTTCCAACTTTG
SEQ ID NO:222 (LA5(4)) GGAATTCGGCCCCCGAGGCCTTCAGCTCCAGCTTGG
SEQ ID NO:223 (LAlambda(1)) GGAATTCGGCCCCCGAGGCCCCTAGGACAGTCAGTTTGG
```

3.2 Establishment of autonomously replicating cell lines by the AGS method

The resulting library was transfected into a packaging cell, Pt-E, (Morita, S. *et al.*, Gene therapy 7, 1063-1066 (2003)) using FuGENE 6 (Roche Diagnostics). Specifically, Pt-E was plated onto 6-cm dishes and cultured in DMEM/10% FBS (Invitrogen). A mixture of FuGENE 6 and the library was added to the plate the following day. The culture medium was exchanged the next day, and the culture supernatant was collected 24 hours after that. 10 µg/mL polybrene (Hexadimethrine Bromide; Sigma) and 2 ng/mL mIL-3 were added to the culture supernatant containing recombinant virus particles. The viral solution was used to infect the BaF3-monkey Mpl target cells. The cells were washed with PBS the following day, and suspended in RPMI 1640/10% FBS minus mIL-3. The suspension was plated onto a 96-well plate at a cell density of 1,000 cells/well. Autonomously replicating cell lines (AB317 and AB324) were obtained after seven days of incubation. Genomic DNAs were extracted from these cells using a DNeasy Tissue Kit (QIAGEN), and the antibody genes were amplified by PCR.

The composition of the PCR reaction solution (50 µL in total) is shown below.

10x LA Taq Buffer (TaKaRa)	5 µL
2 mM dNTPs (dATP, dGTP, dCTP, and dTTP) (TaKaRa)	5 µL
2.5 mM MgCl₄ (TaKaRa)	5 µL
TaKaRa LA Taq (TaKaRa)	0.5 µL
Genomic DNA	0.5 µg
AGSdbS1 (SEQ ID NO: 226) and AGSdbA1 (SEQ ID NO: 227)	25 pmol

The reaction conditions were:
94°C (initial temperature) for 1 min;
30 cycles of 94°C for 30 sec, 60°C for 30 sec, and 70°C for 1 min; and final extension was at 72°C for 6 min.

The nucleotide sequences and the amino acid sequences of the fragments of cloned antibodies are shown below.

Fragment		Nucleotide sequence	Amino acid sequence
AB317	H chain	SEQ ID NO: 154	SEQ ID NO: 155
	L chain	SEQ ID NO: 156	SEQ ID NO: 157
AB324	H chain	SEQ ID NO: 158	SEQ ID NO: 159
	L chain	SEQ ID NO: 160	SEQ ID NO: 161

3.3 Activity assays of the diabodies obtained by AGS method

Each of the anti-Mpl diabodies obtained above was inserted into the pCXND3 expression vector. The PCR primers used are a synthetic oligonucleotide complementary to the 5' end of the diabody and containing an EcoRI site, and a synthetic oligonucleotide complementary to the nucleotide sequence of the 3' end of the diabody and containing a FLAG tag and a NotI site. The PCR product thus obtained was inserted into pCXND3 between the EcoRI and NotI sites. The diabody was expressed transiently in COS7 cells by the method described in Example 2.4. The culture supernatant was removed and the activity of the diabody was evaluated.

The binding activities of the diabodies were assessed by flow cytometry using CHO cells that express Mpl derived from various species (Fig. 8). AB317 was proven to bind to CHO-mouse Mpl.

The TPO-like agonistic activities of the diabodies were evaluated using BaF3-human Mpl, BaF3-monkey Mpl, and BaF3-mouse Mpl (Figs. 9, 10, and 11). AB317 had the highest agonistic activity against human, monkey, and mouse Mpl, whereas AB324 showed the highest agonistic activity against human and monkey Mpl.

This proves that anti-Mpl diabodies having high agonistic activity can be obtained by the AGS method.

[Example 4] Agonistic activity assays of the anti-Mpl antibodies against mutant Mpl in congenital amegakaryocytic thrombocytopenia (CAMT) patients
4.1 Establishment of BaF3 cell lines introduced with the mutant Mpl observed in CAMT patients

Mutations on G305C (R102P), C769T (R257C), and C823A (P275T) have been reported in the Mpl gene of CAMT patients. The respective

expression vectors carrying the Mpl gene mutations were constructed and introduced into BaF3 cells. The following Mpl gene fragments were constructed.

Fragment	Nucleotide sequence	Amino acid sequence
Normal Mpl gene	SEQ ID NO: 246	SEQ ID NO: 123
Mutant Mpl gene, G305C, in which C is substituted for 305th nucleotide G relative to the initiation codon	SEQ ID NO: 247	SEQ ID NO: 248
Mutant Mpl gene, C769T, in which T is substituted for 769th nucleotide C	SEQ ID NO: 249	SEQ ID NO: 250
Mutant Mpl gene, C823A, in which A is substituted for 823rd nucleotide C	SEQ ID NO: 251	SEQ ID NO: 252

5   The above-described DNA fragments were digested with EcoRI and SalI, and inserted between the EcoRI and SalI sites on the animal cell expression vector pCOS2-Ha to prepare pCOS2-hMPLfullG305C, pCOS2-hMPLfullC769T, and pCOS2-hMPLfullC823A.

The genes were introduced into BaF3 cells by the procedure
10   described in Example 1.1 to establish BaF3 cell lines expressing each Mpl gene: BaF3-human MPL (G305C), BaF3-human MPL (C769T), and BaF3-human MPL (C823A). After the selection, the cells were cultured and passaged using RPMI 1640 containing 1 ng/mL mIL-3 and 10% FBS.

15   4.2 Preparation of anti-human Mpl diabody and sc(Fv)2

Among the amino acid sequences shown in Figs. 6 and 7, expression vectors were prepared for the diabodies VB8B, VB45B, VB33, VB140, VB157, and TA136 using the same procedure described in Example 2.2. The prepared expression vectors were introduced into COS7 cells by
20   the same procedure described in Example 2.4. The supernatant concentration of each diabody was determined by the method of Example 2.5. The sc(Fv)2 expression vector for TA136 was prepared by the same procedure described in Example 2.3. The vector was introduced into

CHO-DG44 cells by the same procedure described in Example 2.4. sc(Fv)2 was purified from the culture supernatant thus obtained using the same method described in Example 2.6.

5 4.3 Agonistic activity assays of sc(Fv)2 and the anti-human Mpl diabodies

The prepared diabodies and sc(Fv)2 were assayed for their agonistic activities in normal Mpl and mutant Mpl in BaF3 cells by the same procedure described in Example 2.8. The agonistic

10 activities in BaF3-human Mpl and BaF3-human Mpl (G305C) were compared using the culture supernatants of cells expressing the diabodies. The TA136 diabody (TA136 db) was shown to have a low agonistic activity in BaF3-human Mpl cells expressing the normal Mpl gene, and a high agonistic activity in BaF3-human Mpl (G305C) cells expressing the

15 mutant Mpl gene. hTPO and the rest of the diabodies did not show a high agonistic activity in BaF3-human Mpl (G305C) cells (Figs. 12 and 13).

In addition, the agonistic activities of the TA136 diabody and TA136 sc(Fv)2 in BaF3-human Mpl, BaF3-human Mpl (G305C), BaF3-human

20 Mpl (C769T), and BaF3-human Mpl (C823A) cells were assessed using a purified sample of the diabody. Compared with hTPO and the TA136 diabody, TA136 sc(Fv)2 exhibited a higher agonistic activity in all three types of the TPO receptor mutant cell lines (Figs. 15, 16 and 17). Furthermore, it was shown that in BaF3-human Mpl cells

25 expressing the normal Mpl gene, the TA136 diabody exhibited a lower activity than hTPO. However, an agonistic activity equivalent to that of hTPO was achieved by converting the diabody into sc(Fv)2 (Fig. 14).

30 Industrial Applicability

Various clinical trials had been conducted on recombinant human TPO as a therapeutic agent for thrombocytopenia following chemotherapy. Some clinical trials reported a serious problem, namely, the production of anti-TPO antibodies due to TPO

35 administration (Junzhi Li, *et al.*, Blood 98, 3241-324 (2001); Saroj Vandhan-Raj. *et al.*, Ann. Intern. Med. 132, 364-368 (2000)).

Specifically, the production of neutralizing antibodies which inhibit the activity of endogenous TPO have been reported, triggering the onset of thrombocytopenia. In the present invention, the administration of agonistic minibodies against TPO receptor does not induce the production of antibodies against endogenous TPO. Reduction of the molecular weight of antibodies increases the specific activity of antibodies and shortens the half-life in blood. Thus, the effective concentration of an antibody in blood can be easily controlled, presenting an advantage in clinical applications. Accordingly, such an antibody can be used as an agent to treat thrombocytopenia more effectively than the naturally-occurring TPO or its agonistic antibodies. Since minibodies are not attached with sugar chains, the expression systems for expressing those recombinant proteins are not limited, and minibodies can be prepared by using any of the expression systems derived from mammalian cell lines, yeasts, insect cells, and *E. coli*. In addition, minibodies have a binding affinity towards mutant TPO receptor different from that of TPO. Therefore, minibodies are expected to bind and exhibit agonistic activities against specific TPO receptor mutants, which contain mutations commonly detected in CAMT patients with thrombocytopenia and genetically mutated TPO receptors.